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Master´s Degree

Medicine and Molecular Oncology

K14-HPV16 mouse model: A journey towards early HPV-induced head and neck *versus* anal, penile and uterine carcinogenesis

Diogo Miguel Monteiro Estêvão

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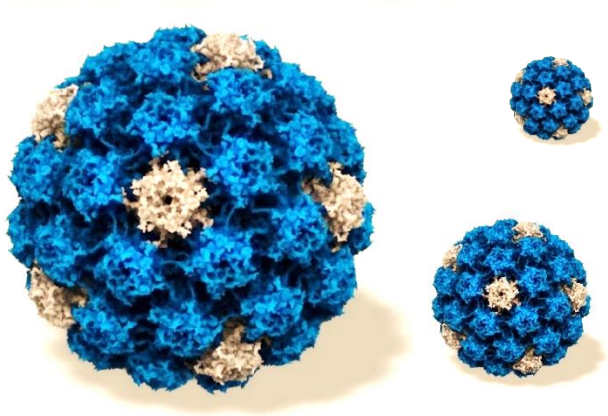

The Dissertation was submitted to the Faculty of Medicine of the University of Porto as an integrated part of the Master´s Degree in Medicine and Molecular Oncology.

Faculty of Medicine

University of Porto

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The research work presented in this master´s dissertation was carried out at the Molecular Oncology and Viral Pathology Group in the Portuguese Institute of Oncology, (IPO-Porto), under the supervision of Professor Rui Medeiros, Portuguese Institute of Oncology (IPO-Porto) and the co-supervision of Doctor Rui Gil da Costa, Center for the Research and Technology of Agro-Environmental and Biological Sciences (CITAB), University of Trás-os-Montes and Alto Douro (UTAD) and Doctor Natália Costa, Portuguese Institute of Oncology (IPO-Porto).

Faculty of Medicine

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Knowing is not enough we must apply. Willing is not enough we must do.

Goethe

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Abstract

Introduction/Aims: Human papillomavirus (HPV) is the most common sexually transmitted agent worldwide, being also responsible for 5% of all human cancers. Even though cervical cancer's incidence is decreasing, HPV-driven anogenital and head and neck cancers are increasing. Differences in the natural history of HPV have been observed by gender and anatomic sites of infection, where genetic expression, hormonal responses, epithelial backgrounds and tissue stromal microenvironments may play a crucial role. The main goal of this study was to observe if E6, E7 and E5 HPV oncoproteins could trigger distinct grades in the HPV-related carcinogenic pathways in the K14-HPV16 mouse model, on the most affected human anatomic sites of infection namely in the uterine cervix, base of tongue, anus, penis and bladder.

Methods: Samples from the base of the tongue, anus, uterine cervix and bladder were collected from ten K14-HPV16 female mice, 30-weeks-old. Alongside, samples from the base of the tongue, anus and penis were collected from ten K14-HPV16 male mice, 30-weeks-old. Matched samples of E6, E7 and E5 mRNA levels were quantified by real-time PCR, after normalization using the best two housekeeping genes. Histopathological analysis of the tissues was performed for tissue characterization. Tissue samples were classified as normal, low-grade squamous intraepithelial lesion, high-grade squamous intraepithelial lesion and squamous cell carcinoma. Statistical analysis was performed using the IBM SPSS Statistics. Kruskal-Wallis and Mann-Whitney tests were used to evaluate statistical differences in normalized relative expression ($-\Delta Ct$) of the *E6, E7* and *E5* genes among the different tissue samples.

Results: The expression pattern of the oncogenic HPV viral mRNAs E6, E7 and E5 was similarly detected across tissues ($p>0,05$), except in the bladder ($p< 0.01$). However, we observed a higher incidence of more advanced lesions, namely high squamous intraepithelial lesions and squamous cell carcinoma in the base of the tongue when compared with the other studied anatomic sites. In uterine cervix and bladder samples, only normal epithelium was observed. Alongside, in the base of the tongue, the female K14-HPV16 developed 50% more squamous cell carcinoma than their male counterparts.

Conclusion: This study suggests that the carcinogenesis induced by HPV in the oropharyngeal region is earlier and less dependent on other external co-factors being more incident in females . In the base of the tongue, cancer was induced within the mice 30 weeks period, in comparison with the other anatomic locations, where HPV itself seems not to be sufficient to promote more advanced lesions even though the expression of the viral mRNAs was similarly detected within the tissues. Future studies should focus on understanding the behaviour of the HPV oncoproteins and the related

oncogenic pathways at multiple anatomic locations of infection, representing different tissue microenvironments. This might allow a better understanding of tissue-specific HPV-related carcinogenic steps and the development of precision therapies.

Keywords

HPV, oncoproteins E6, E7 and E5, K14-HPV16 transgenic mouse model, HPV-induced cancer

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Abbreviations

APC	Antigen presenting cells
ASR	Age-standardized incidence rate
Bp	Base pairs
β2m	Beta-2-microglobuline
CCNAI	Cyclin A
CCNEI	Cyclin E
cDNA	Complementary deoxyribonucleic acid
CRISPR-Cas9	Clustered regularly interspaced short palindromic repeats-associated protein 9
CR1	Conserved region 1
CR2	Conserved regions 2
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
E6-AP	E6-Associated protein
HDACs	Histone deacetylases
HGSIL	High-grade squamous intraepithelial lesion
HNSCC	Head and neck squamous cell carcinoma
HPV	Human papilloma virus
HSPGs	Heparan sulfate proteoglycans´
HD5	Human α-defensin
IARC	International agency for research on cancer
K	Keratinocytes
KDa	Kilodalton
LC	Langerhans cell
LCR	Long control region

LGSIL	Low-grade squamous intraepithelial lesion
MHC	Major histocompatibility complex
MMP	Metalloproteinase
mRNA	Messenger ribonucleic acid
miRs	MicroRNAs
NTC.	No template control
OPSCC	Oropharyngeal squamous cell carcinoma
ORF	Open reading frame
PCR	Polymerase chain reaction
PV´s	Papilloma virus
Q1	Quartile 1
Q3	Quartile 3
pRB	Retinoblastoma protein
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RT-PCR	Real-time polymerase chain reaction
SIL	Squamous intraepithelial lesion
TBP	TATA-box binding protein
TFIID	Transcription factor TATA binding protein
UV	ultra-violet
VLP	Virus like-particle

Publications

The results presented in this dissertation are either published or have been submitted for publication in peer-reviewed scientific journals as well as presented for oral communications.

- Estêvão D. *et al.* CRISPR-Cas9 therapies in experimental mouse models of cancer. Future Oncology. 2018 July.

- Rui M. Gil da Costa, Tiago Neto, Diogo Estêvão *et al.* Ptaquiloside from bracken (Pteridium spp.) promotes oral carcinogenesis initiated by HPV16 in transgenic mice. Life Sciences (submitted).

- Estêvão D. *et al.* Hallmarks of HPV carcinogenesis**:** the role of E6, E7 and E5 oncoproteins in cellular malignancy. BBA Gene Regulatory Mechanisms (submitted review)

- Estêvão D. *et al.* K14-HPV16 Mouse Model: A journey towards early HPV-induced head and neck vs anal and uterine carcinogenesis. Journal of Virology (submitted)

- Rui M. Gil da Costa, Diogo Estêvão *et al.* HPV16 induces penile intraepithelial neoplasia and penile squamous cell carcinoma in transgenic mice: a mouse model for penile cancer (paper under preparation).

- "K14-HPV16 mouse model: A journey towards early HPV-induced head and neck versus anal and uterine carcinogenesis", Oral communication in the EUROGIN 2018, Lisbon, Portugal (Annexes).

- "K14-HPV16 mouse model: A journey towards early HPV-induced head and neck versus anal, uterine and bladder carcinogenesis", Poster secion in the AIMS 2019 Conference, Lisbon, Portugal (Annexes).

General Introduction

I. General Introduction

1.1. Human papillomavirus and cancer

Viruses are among the most prominent risk factors for cancer development (1). Human papillomavirus (HPV) itself is the most common sexually transmitted agent worldwide, afflicting 50-80% of the sexually active human population (2). It is also responsible for one-third of all the tumours induced by viruses and accounts for 5% of all human cancers, being one of the most powerful human carcinogen (1). This translates in approximately 630.000 new HPV-related cancer cases per year, being therefore, a huge public health burden (3).

Since HPV identification as the cause of cervical cancer, achieved by Harald Zur Hausen, who was awarded the 2008 Nobel Prize in Physiology/Medicine, this area has been an intensive focus of research. Hausen and colleagues pursued the idea that if tumour cells contained an oncogenic virus, they should harbour viral deoxyribonucleic acid (DNA) integrated into their genome (4). In 1983, Hausen found integrated HPV-DNA not only in genital cancer biopsies but also in cell lines derived from cervical cancer, discovering the high-risk HPV16 (4,5) **(Figure 1)**.

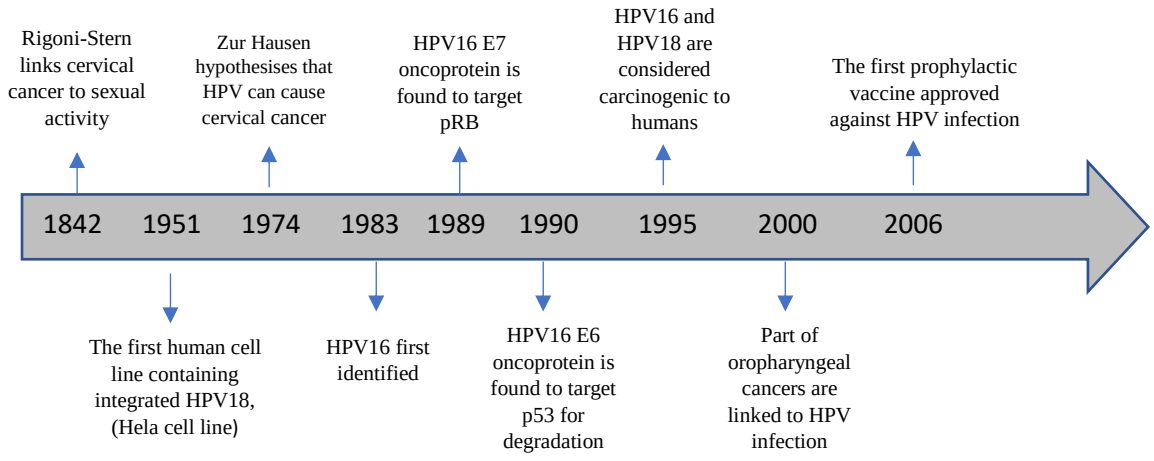


Figure 1. HPV main milestones.

1.2. HPV Biology

1.2.1. HPV genome

HPVs are a small, double-stranded and non-enveloped virus with 50-55 nm in diameter (6). Their episomal genome has approximately 8000 base pairs, mostly encoding 8 open reading frames (ORFs), expressed from polycistronic messenger ribonucleic acid (mRNA) (6) (**Figure 2**).

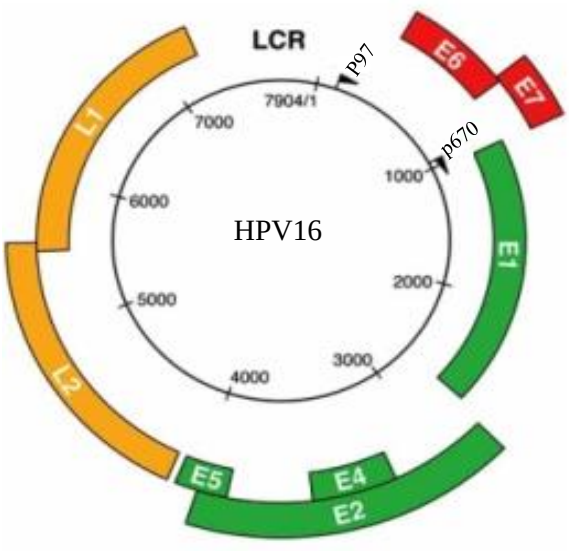


Figure 2. HPV16 episomal genome. HPV16 genome can be grouped into three main regions: 1) An early region, encoding the early proteins E6, E7, E1, E2, E4, E5; 2) a late region, encoding the late proteins L1 and L2 and 3) a long control region (LCR), that comprises the origin of replication and influences several transcriptional factors. Adapted from (7) .

HPV infects the basal keratinocytes, being its expression temporal and synchronized with their differentiation (7). It expresses early and late proteins which are named based on their temporal expression in the epithelium. The early region, denoted by "E" which occupies approximately 50% of the HPV viral genome, encodes the early proteins E6, E7, E1, E2, E4 and E5 which are crucial for viral replication, maturation and transformation (7) (**Figure 2**). The late region, denoted by "L", which occupies around 40% of the viral genome, encodes the major capsid protein L1 and the minor capsid protein L2 that are structural viral proteins vital for virus assembly and release (7) (**Figure 2**). Finally, the long control region (LCR), covering only 10% of the HPV genome, contains 1) the viral promoter *p97*, crucial for the expression of the early viral proteins, 2) the origin of viral DNA replication, 3) several binding sites of transcription factors like SP-1, AP-1 and the transcription factor TATA binding protein (TFIID), essential in the regulation of viral expression and 4) cis-sequences that regulate mRNA stability, polyadenylation and numerous alternative splicing mechanisms (8) (**Figure 2**). In the *E7/E1* ORF region, a *p670* promotor is found, which is important in encoding the viral mRNA during the HPV genome amplification and viral escape steps in the upper layers of the epithelium (7) (**Figure 2**).

1.2.2. HPV evolution

HPVs belong to the Papillomaviridae family, one of the most ancient viral families, with at least 350 million years old, affecting a diverse range of animal species, including humans (9) (**Figure 3**). HPV is highly heterogeneous and according to the American Cancer Society, more than 300 genotypes of papillomavirus (PVs) have been identified, 200 of them capable of infecting humans. Nevertheless, HPV is highly conserved, showing a divergence rate of 1% at every 40.000-80.000 years (10).

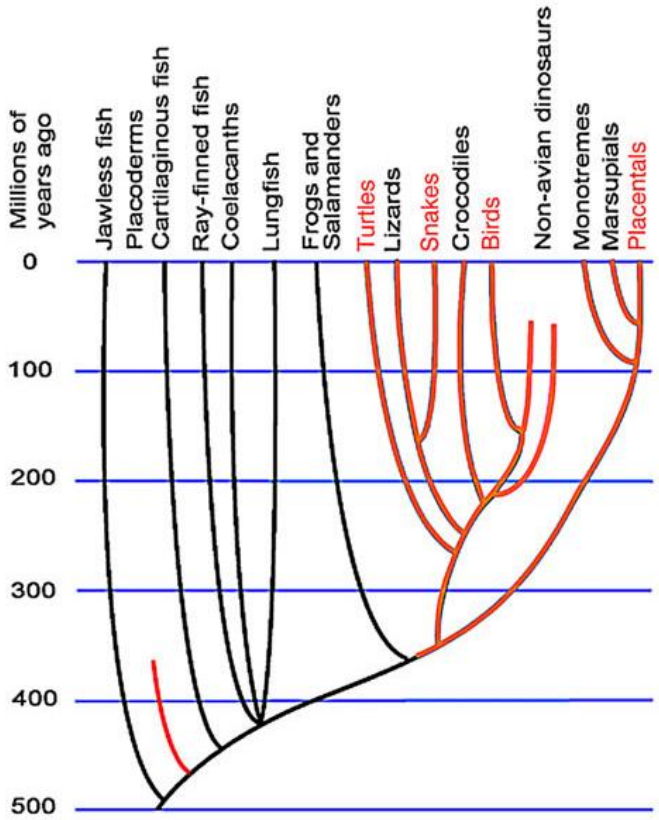


Figure 3. Papillomavirus evolutionary tree with a 350 million years old common ancestor of reptiles, amphibious and mammals. Adapted from (11).

HPV is grouped within 5 evolutionary branches, named according to the Greek alphabet, namely the alfa, beta, gamma, nu and mu papillomaviruses (α, β, γ, μ, and ν respectively) (**Figure 4**). The largest and the clinically most important HPV group, the α-HPV, comprises 64 HPVs, being able to infect the reproductive, oral and anogenital mucosae and therefore responsible for the development of high-grade lesions that can progress to cancer (12).

The α-HPV can be subdivided into two major subgroups, namely the low-risk HPVs (e.g. HPV6 and 11) mainly associated with the development of warts and benign lesions (13) and the high-risk HPVs (e.g. HPV16 and 18) which are considered the main drivers of high-grade lesions' development and consequently cancer progression due to its stronger binding and function to its molecular targets leading to a higher differential and transformation properties (12). HPV16 itself has a great ability to persist and promote malignant transformation, higher than any other HPV type. It is also the most prevalent HR-HPV being found in approximately 50% of the cervical cancers and more than 85% of HPV-induced anal and head and neck cancers (14).

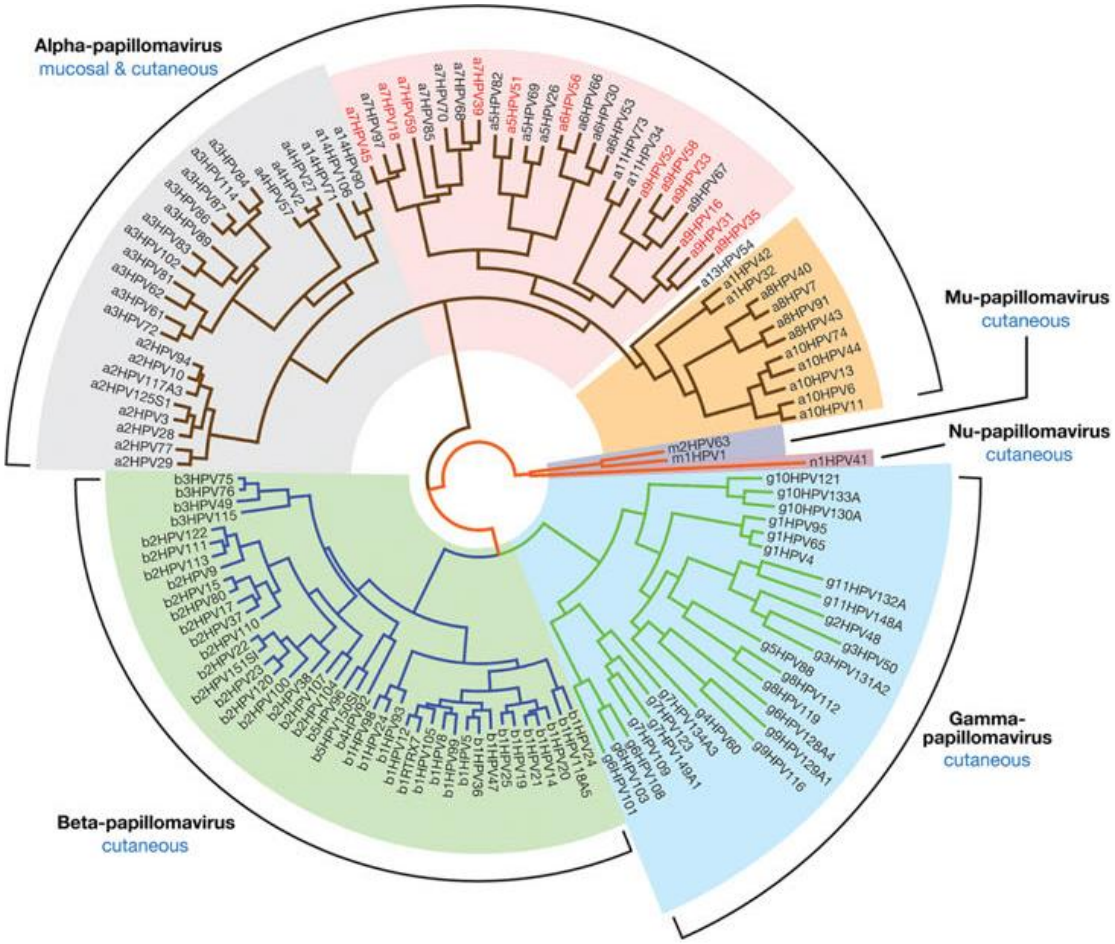


Figure 4. Evolutionary relationships between the HPV genera, namely the alfa, beta, gamma, nu and mu papillomavirus. Adapted from (12).

1.3. HPV viral proteins

1.3.1. Main drivers of oncogenesis

Even though all genes encoded by HPV are necessary for its life cycle and regulation, the E6, E7 and E5 oncoproteins are essential for the malignancy process of HPV-positive carcinomas (15). Therefore, it is crucial to identify and understand how these viral oncoproteins contribute to the all known cancer cell alterations allowing the progression of the carcinogenesis (15) (**Table 1**). (Hallmarks of HPV carcinogenesis: the role of E6, E7 and E5 oncoproteins in cellular malignancy. BBA Gene Regulatory Mechanisms, submitted review).

1.3.1.1. E6 oncoprotein

The E6 protein is one of the well-established and recognized oncoproteins that are associated with the malignant progression of HPV-infected cells. It has 18 kDa and 2 zinc-binding motifs, being mainly found in the nucleus (16). The HPV oncoproteins can abrogate the apoptosis mechanisms, allowing the infected cells with genomic errors to resist cell death. From all the roles promoted by the E6 oncoprotein, the most important and studied is the degradation of the p53 *guardian of the genome* protein, a transcription factor that regulates apoptosis, DNA repair and the cell cycle (17). The E6 oncoprotein targets and interacts with the conserved *LxxLL* consensus sequences of the Ubiquitin-protein ligase E3A (UBE3A) also known as ubiquitin ligase E6-Associated Protein (E6-AP), that works as a connected bridge between the E6 oncoprotein and its primary target, the p53, targeting it for proteasomal degradation (**Figure 5**) (17). Other cellular targets of E6 have also been identified (**Table 1**).

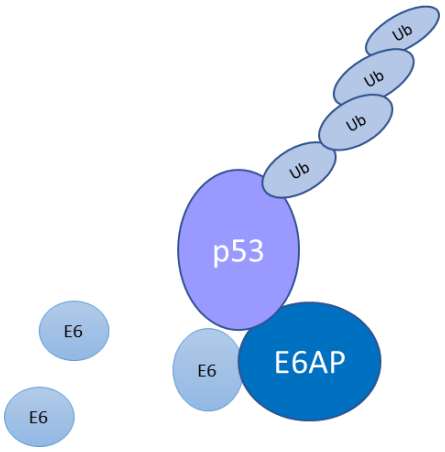


Figure 5. Ubiquitination and consequent proteasomal degradation of p53 by the formation of a complex ternary E6-AP; E6; p53.

1.3.1.2. E7 oncoprotein

E7 is a small phosphoprotein with approximately 16 kDa and three conserved regions (CR1, CR2 and CR3) (18). It was the first HPV malignant oncoprotein to be discovered, being found in the nucleus (19). The CR2 regions of E7 contain an *LXCXE* motif that is important in the association of its transforming targets, namely the retinoblastoma protein (pRB), a regulator of the cell cycle control (20). Expression of E7 leads to the degradation of the pRB tumour suppressor protein targeting it for proteossomal degradation through the ubiquitonation pathway, deregulating cellular replication (20) (**Figure 6**). The real damage of the unbalanced expression of E6 and E7 viral proteins happens when these two proteins are expressed simultaneously, leading to cell immortalization (21,22).

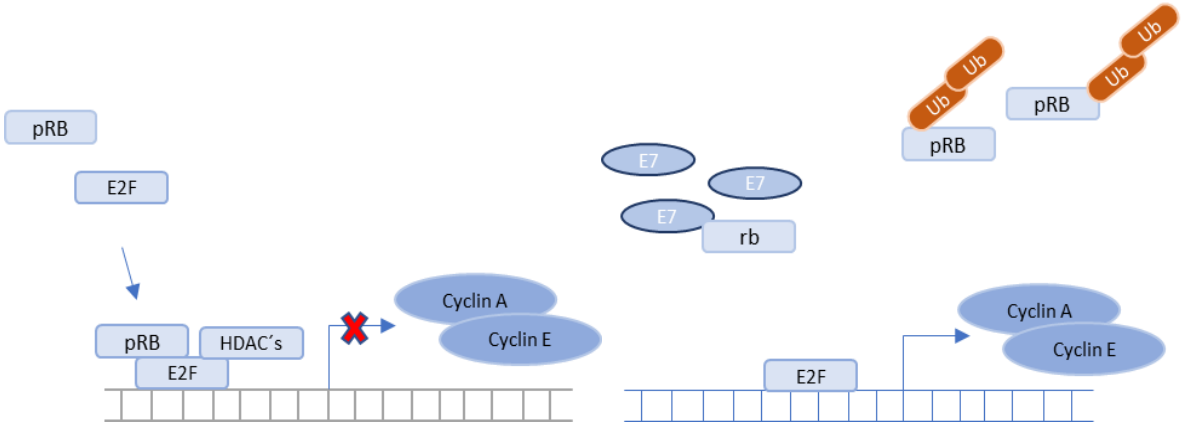


Figure 6. Cell cycle regulation of pRB and E2F in a) normal cells and in b) HPV-infected cells. A) In normal cells, during the G1 phase, the unphosphorylated pRB protein binds to the transcription factor E2F and concomitantly recruits histone deacetylases (HDACs) to target promotor sequences of E2F, therefore repressing the expression of proliferating genes, cyclin A (CCNA1) and cyclin E (CCNE1). b) in HPV infected cells, E7 targets the pRB protein for degradation, inhibiting the binding with the transcription repressor E2F and consequently not recruiting the HDACs, allowing the transcription of the proliferating genes (23).

1.3.1.3. E5 oncoprotein

Although the major transforming activities in the HPV-related lesions are led by the oncoproteins E6 and E7, the 10 kDa transmembrane protein E5, mainly localized in the intracellular membranes of the endoplasmic reticulum and Golgi apparatus, also plays an important role (24). The oncogenic properties of E5 protein have always been limited, however, recent studies have shown its pivotal importance, particularly in cell transformation and immune modulation, that together with oncoproteins E7 and E6 helps in the cell malignant progression (25). One of the most important roles of E5 oncoprotein is its interaction with the major-histocompatibility-complex (MHC), important in the activation of the immune system (26).

E5 promotes the retention of the MHC class I in the Golgi apparatus, inhibiting its transportation to the cell surface, fact that leads to a decreased ability of the complex to recognize the HPV viral antigens and therefore activate the immune system (26). Other functions in the HPV viral life cycle and in the malignant progression have also been identified (**Table 1**).

Table 1. Main functions and targets of E6, E7 and E5 HPV oncoproteins. ↓ Downregulation; ↑upregulation; ✖ action blocking

High-risk Viral protein	Cellular location	Function	Main targets	Expression level	References
E6	Nucleus and cytoplasm	Escaping cell death	P53 protein	↓	(17)
			Pro-caspase 8 protein	↓	(27)
			Bak protein	↓	(28)
			TNR1	✖	(29)
			Fas/Fas ligand death pathway	✖	(30)
			NF-κB; cIAP-2	↑	(31)
		Deregulation of cell cycle	P300/CBP complex protein	↓	(32)
			miR34a	↓	(33)
		Immune system modulation	IRF3	↓	(34)
			IFNα	↓	(35)
			IFNκ	↓	(36)
		Cell Immortalization	NFX1-91	↓	(37)
			Myc	↑	(38)
			Sp1	↑	(39)
		Genomic instability	APOBEC3	↑	(40)
			XRCC1	↓	(41)
		Cell invasion	Dlg	↓	(42)
			SCRIB	↓	(43)
			MAGI-1, MAGI-2 and MAGI-3	↓	(44)
			PAR3	↓	(45)
			Fibulin-1	↓	(46)
			miR-23b	↓	(47)
			Paxillin disruption	↓	(48)

E7	Nucleus	Deregulation of cycle cell	pRB protein	↓	(49)
			p107/p130	↓	(49)
			P21	↓	(50)
			P27	↓	(50)
			Claspin	↓	(51)
			E2F6	↓	(52)
		Immune system modulation	TLR9	✘	(53)
			Cgas-STING	✘	(54)
		Genomic instability	Abnormal centrosome synthesis	✘	(55)
			γ-tubulin	✘	(56)
			CDK2	↑	(57)
E6/E7	Nucleus and cytoplasm	Deregulation of cellular energetics	Aerobic glycolysis	↑	(58)
			mTORC1	↑	(59)
			GLUT-1	↑	(60)
		Genomic instability: epigenetic deregulation	DNMT1 DNMT3A DNMT3B	↑	(61)
			E-cadherin	↓	(62,63)
			CXCL14	↓	(64)
			CCNA1	↓	(65)
			CBP/p300	↓	(66)
			TIP60	↓	(67)
			ADA3	↓	(68)
		Inflammation promotion	IL-6	↑	(69)
			IL18	↑	(70)
		Angiogenesis switch	Maspin	↓	(71)
			Thrombospondin-1	↓	(71)
			VEGF	↑	(71)
			IL-8	↑	(71)
E5	Endoplasmic reticulum and Golgi apparatus	Sustaining Proliferative signalling	EGFR	↑	(72)
			ATPase	↑	(73)
			KGFR/FGFR2b	↓	(74)
		Escaping cell death	Bax protein	↓	(75)
		Cell invasion	MET	↑	(76)
		Immune system modulation	MHC-class I	✘	(26)

1.3.2. Other HPV viral proteins

HPV expresses other early and late proteins that are essential for its life cycle such as early viral proteins E2, E1 and E4 and the late viral proteins L1 and L2. Their functions will be briefly mentioned.

1.3.2.1. E2 viral protein

The E2 protein has approximately 48 kDa, being mainly found in the nucleus (77). It is important not only in the recruitment of the E1 protein to the HPV origin site of replication but also in the regulation of E6 and E7 expression (78). This protein, particularly in the initial stages of HPV infection, binds to the transcription sites near the *E6* and *E7* promoter regions, downregulating their expression to a low copy number, just enough to push cells to be mitotically active, but not sufficient to activate the immune system (79). When *E2* is disrupted, mainly by HPV integration in the host genome but also by hypermethylation mechanisms, the negative feedback of this protein onto the E6 and E7 genomic region is repressed (78). This fact leads to an unbalanced expression of E6 and E7 oncoproteins and consequent oncogenic transformation of the infected cells, that has been shown to be reversed by E2 reintroduction into cervical carcinoma cells (80).

1.3.2.2. E1 viral protein

The E1 viral protein has about 70-80 kDa in size, being the largest papillomavirus protein (81). It is probably the most important protein in the HPV life cycle since it is essential for the initiation of viral DNA replication (82). It is also the only HPV protein with enzymatic activity (82). It has the main function of encoding a DNA helicase, unwinding DNA and consequently making it accessible for cellular replication proteins. Furthermore, it also has ATPase activity and the ability to recruit host cells' replication proteins like DNA polymerases, topoisomerases I and alpha-primases (82,83).

1.3.2.3. E4 viral protein

The E4 protein, with 17 kDa, plays an important role in maturation and virus escape from the epithelial surface (84). E4 viral proteins are mainly found in the cytoplasm, but can also be present in the cell nucleus (84). It is also believed that this protein can promote the collapse of the cellular cytokeratin network, facilitating the viral escape (85). Although the E4 viral protein is considered an early protein, its function and expression are mainly associated with later stages of the infection.

1.3.2.4. L1 major capsid protein

The major capsid L1 protein, with 55 kDa, comprises 72 pentamers and is essential in capsid formation and self-assembling into a virus-like-particle (VLP), forming a virion that contains 360 copies of the L1 protein (86). L1 protein is also important in the interaction of HPV with the target basal cell, leading to viral cell entry (87). Due to its pivotal role in the formation of viral capsids as well as its high conservation status, it has been the main target in the development of HPV prophylactic vaccination (88).

1.3.2.5. L2 minor capsid protein

The L2 minor capsid protein has 80 kDa, being highly conserved (89). L2 structural viral protein not only plays a key role in virus assembly and infection but also in the transportation of the viral particles to the cytosol as well as the HPV genome to the nucleus (90).

1.4. HPV infection pathway

HPV is highly epitheliotropic, only establishing a productive infection in the stratified epithelial keratinocyte cells, being rarely detected in other types of cells (12). It targets the basal keratinocytes stem cells, the only ones that are able to actively divide (12). These infected keratinocytes stem cells are mainly found in the bulge of the hair follicle or even at the transformation zone in the uterus between the ecto- and the endocervix, assuring a continuous reservoir of HPV infected cells (91).

The probable cause of infection is the existence of micro-lesions in the reproductive, anogenital and oral area, being the infection transmitted mainly during sexual activity (92). However, HPV vertical transmission from mother to child can also be possible (93). Even though most sexually active people will be infected by HPV at some part of their life's, most of these infections will be cleared by their immune system within a period from 24-36 months (88).

1.4.1. Keratinocyte-dependent infection

The epithelia is comprised of several types of keratinocytes (K), that are found in the different layers of the epithelium, comprehending 90% of all epidermis cell types (94). While K14 and K5 are found in the basal membrane, in the spinous and granular epidermis layers, K1 and K10 are the most common ones (95). Keratinocytes can have two fates: they can be stem cells' keratinocytes, having the capability of undefined self-renewal, or can be basal transit amplifying keratinocytes cells. The former ones, after mitotic activity, migrate upwards in the epithelia, becoming differentiated and forming the upper layers of the epidermis, ensuring skin stability and protection against the exterior environment (96). However, the suprabasal HPV-infected cells fail to withdraw from the cell cycle as well as to become completely differentiated, continuing to proliferate due to the HPV viral expression.

1.4.2. HPV binding and endocytosis

Initially, HPV, through the L1 protein, targets the basal keratinocytes by binding to the glycoproteins heparan sulfate proteoglycans´ (HSPGs) receptor, found on the surface of most cells (97). This binding will promote the first conformational change in the HPV viral capsid (98). Posteriorly, the first conformation change will lead to the N-terminus of the HPV L2 protein to be cleaved by furin, which consequently exposes the L2 binding site to a more specific secondary cellular receptor, which is not yet identified, having as possible candidates the Epithelial Growth Factor Receptor (EGFR) or the integrin α-6 on the membrane of the target cell (99). This last mechanism is decisive for viral particles´ internalization, leading to the activation of the pathways necessary for HPV endocytosis (100) (**Figure 7**).

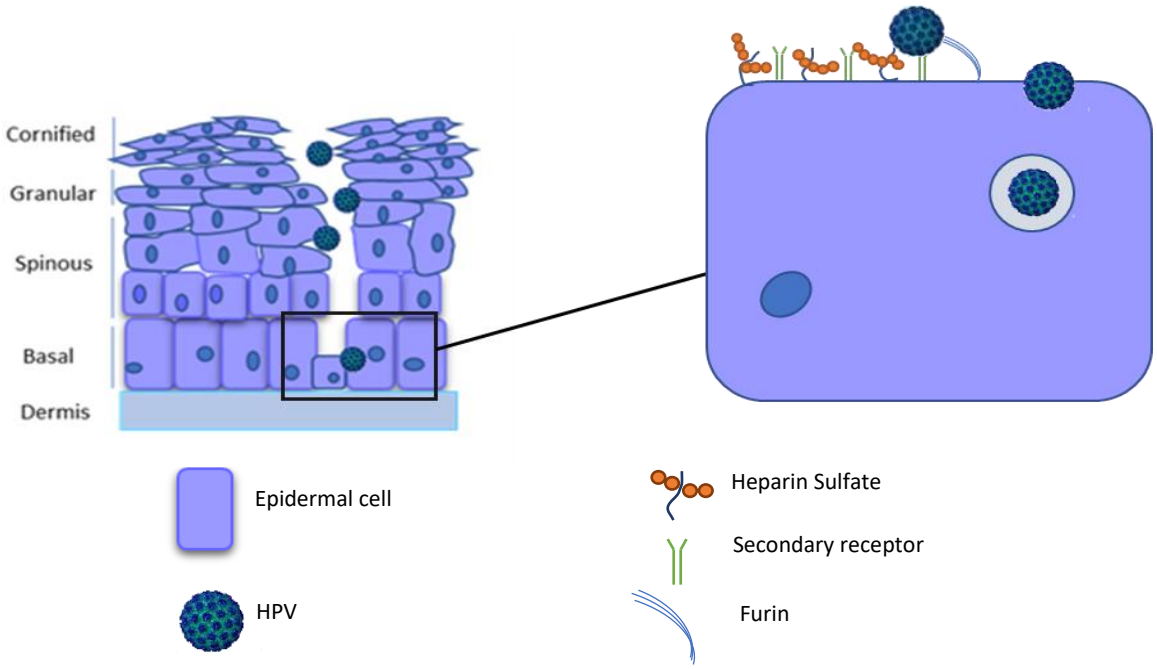


Figure 7. Infectious pathway of HPV. Due to the existence of micro-lesions, HPV infects the cutaneous and mucosal sites in several anatomic regions. Initially, HPV binds to the HSPGs at the basal membrane of the cells, resulting in the first conformation change in the HPV capsid. Posteriorly, the furin protein will cleave the N-terminal region of the L2 minor capsid protein, leading to a second transformation change in the capsid. The L2 viral protein will then be exposed to a secondary receptor, leading to viral internalization, which happens either by clathrin or caveolin-mediated endocytic mechanism.

1.4.3. Virus uptake and nucleus delivery

HPV is internalized by a clathrin or caveolin-mediated endocytic mechanism dependent on the L1 viral protein (101). The most recent studies enlighten that after cell internalization, HPV travels through the endosomal organelles from the early to the late endosomes (102). These organelles, characterized by a low pH, promote viral coat disassembly and L1 and L2 dissociation (87). HPV then travels through the trans-Golgi network and the endoplasmic reticulum until it finally reaches the nucleus (87). The HPV entry into the nucleus occurs by nuclear envelope breakdown in prometaphase, where the viral DNA associates with the condensed chromosomes using the microtube spindle (103).

1.5. HPV life cycle

1.5.1. Basal HPV replication

After a successful infection and travel to the nucleuos, HPV is extrachromosomal, depending on cellular replication to encode the genes for its life cycle. The first viral genes to be expressed, in basal cells, although at a low copy number (50–100 copies per cell), are E6 and E7 oncoproteins (79). These oncoproteins are important in pushing the cells to be mitotically active mainly by the impairment of important regulatory cellular pathways (104,105) (**Table 1**). Alongside, expression of E1 and E2 proteins occurs and this step is crucial to the highjack of the host cellular replication molecules (82,83).

1.5.2. HPV genome amplification

Posteriorly, in the middle-upper epithelial layers, as HPV-infected keratinocytes undergo differentiation and lose their mitotic activity, the HPV late promoter *p670* becomes activated (7). This promoter increases the expression of the HPV viral proteins (at least 1000 copies per cell), leading to viral DNA amplification (7). The HPV genome amplification step is crucial to ensure that the differentiated cells, as they move upwards the epithelium, are sustained in the S-phase like state, allowing HPV to continue its life cycle (79).

1.5.3. Virus release

In the last phase of the HPV life cycle, a higher expression of the early protein E4 and the late proteins L1 and L2 occurs in the upper layers of the epithelium, allowing not only the collapse of the cellular cytokeratin structure but also the packing of HPV genome in infectious virions (85,86). This step is coincident with the shedding of the more mature keratinocytes, which leads to virus release (7). HPV infection is, therefore, an orchestrated event of differential gene expression that ensures smooth progression and limits immune system activation (**Figure 8**).

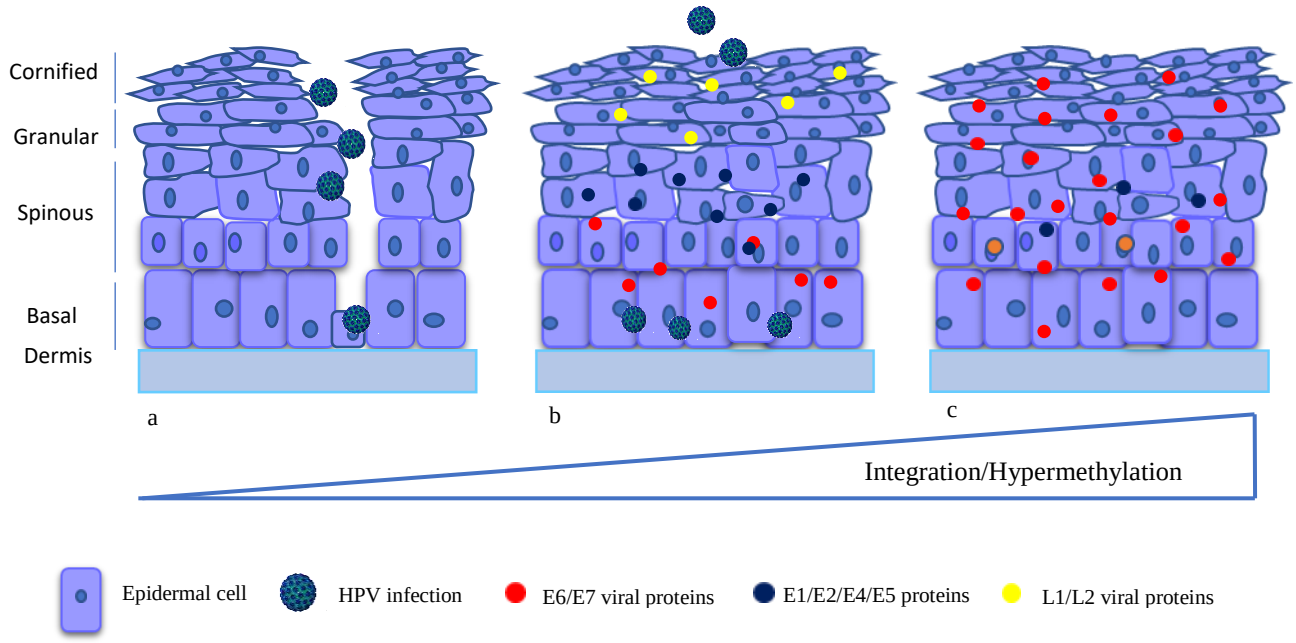


Figure 8. HPV life cycle. a) HPV infects the basal layer of the epithelium through micro-lesions b) At this level, HPV is episomal, depending on cellular replication for encoding the necessary genes for HPV life cycle. E6 and E7 expression is needed throughout the infection, pushing cells to be mitotically active. Additionally, expression of E1, E2, E4 and E5 is crucial for viral amplification. L1 and L2 proteins are expressed in upper epithelial layers c) Accidental integration or hypermethylation mechanisms leads to an unbalanced expression of E6 and E7 and consequently to the development of high-grade lesions that can progress to cancer.

1.6. HPV carcinogenesis

1.6.1. HPV integration

The carcinogenesis driven by HPV infection is not the aim but rather the result of two mechanisms that not only represent a "dead end" for the virus but also to the development of lesions. These two mechanisms englobe an accidental viral DNA integration in the host genome, potentially triggered by the genomic instability caused by the expression of the viral oncoproteins E6 and E7, which increases the double strand break incidence in the host cells (106,107). Even though HPV integration can happen across all the human genome, it is more common in chromosomal fragile regions such as *3q28, 4q13.3, 8q24.21, 13q22.1 17q21* regions, near clusters of microRNAs as well as microhomology regions (1-10 bp) between the host and the virus (108,109).

In the HPV genomic region, the *E2* ORF is usually the most affected location by the integration process, however other genomic regions like E1 as well as L1 and L2 can be affected, leaving E6 and E7 oncoproteins as the primary products expressed by the infected cells which will promote a selective growth advantage not only due to the enhanced expression of the HPV oncoproteins but also because of the altered expression of key cellular genes and changes in the methylation patterns, conferring competitive advantage (110). Alongside, the disrupted *E2* HPV genomic region will also cease to act as a negative regulator of *E6* and *E7*, promoting their unbalanced expression (78). Additionally, mechanisms of HPV hypermethylation were found to block the access of the *E2* promotor region, leading to an unbalanced expression of the *E6* and *E7* viral oncogenes without *E2* disruption (111). Hypermethylation of E2 binding regions can be found in approximately 20% of the cervical cancers and 60% of the HPV-positive oropharyngeal cancers, in which the virus remains episomal and the *E2* genomic region is intact (111,112).

1.7. HPV-derived lesions' classification and staging

Persistent high-risk HPV infection can lead to the transformation of the epithelium cells into pre-cancerous lesions (104). Those lesions are just the ultimate steps before full malignant transformation and consequently the development of squamous cell carcinoma (SCC). Squamous intraepithelial lesion (SIL) is the commonly used term to describe lesions related with HPV persistent infection.

1.7.1. Low-Grade Squamous Intraepithelial Lesions

In low-grade squamous intraepithelial lesions (LGSIL), the epithelium is abnormal with slight changes in cell size, shape, and number. However, it continues to be well differentiated with no nuclear malformations (113). LGSIL are not considered true precursors of cancer but rather the results of the HPV persistent infection. These lesions have a higher probability of regression to normal epithelium, (≈70%) and only 10% may progress to more advanced lesions (114) (**Figure 9**).

1.7.2. High-grade Squamous Intraepithelial Lesions

The high-grade squamous intraepithelial lesions (HGSIL), are defined by a higher number of precancerous cells on the surface of the epithelium, affecting approximately two-thirds of it (113). They have a higher potential of becoming cancerous cells and invade deeper tissues (114) (**Figure 9**). During the timeline in which pre-cancerous lesions are progressing, DNA mutations in oncogenes and tumour suppressor genes, chromosomal aberrations, aberrant DNA methylation and overexpression of oncogenic miRNAs pave the way for the full malignant transformation (115).

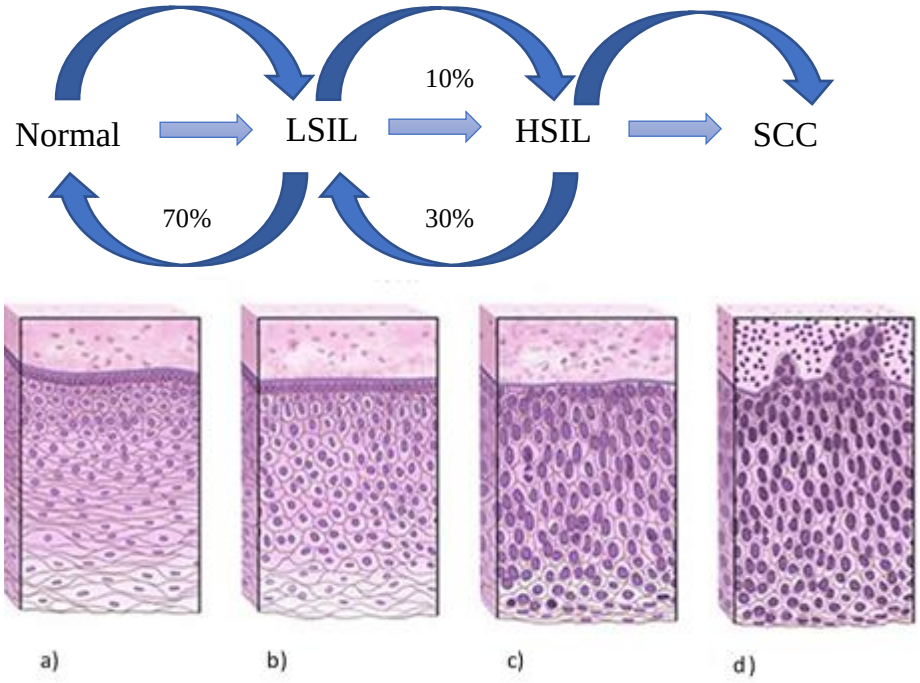


Figure 9. Cancer Lesions progression. a) Normal epithelium, b) LSIL epithelium, with slight changes in cell proliferation and shape, affecting approximately one third of the epithelium. c) HSIL epithelium with abnormal development of cell growth and differentiation as well as nuclei enlargement and nuclear membrane more prominent, affecting approximately two thirds of the epithelia. d) cancerous epithelium with complete cell undifferentiation and capability of invading deeper tissues.

1.8. HPV-induced carcinogenesis factors and co-factors

Persistent HPV infection is the most important risk factor for the development of HPV-derived lesions. However, HPV alone, in some cases, seems not to be a sufficient cause for the full development of a malignancy, fact explained by the small percentage of women that develop cervical cancer when compared to its infectious rate. Other factors must be present to potentiate full malignancy.

1.8.1. Viral factors

The development of HPV-derived cancers is dependent on viral factors themselves, namely the HPV type that infects the epithelium. The high-risk HPVs, particularly HPV16 and 18, pose a greater risk for the development of HGSIL due to their oncogenic potential and prevalence (10). Alongside, the high-risk HPV oncoproteins E6, E7 and E5 play a crucial role in the development of lesions, impairing key regulatory functions in cell cycle regulation, apoptosis and in the immune system (**Table 1**) (104).

1.8.2. External co-factors

1.8.2.1. High parity

External co-factors also play a significant role in HPV-derived carcinogenesis. High parity is consistently considered a risk factor for the development of cervical cancer (116). In fact, a woman that had seven or more full pregnancies has a four-fold increased probability of developing this type of carcinoma (116). Several reasons may explain this difference. During pregnancy, the cervix transformation zone has a higher susceptibility for an infection as well as the hormonal changes that can also modulate the progression of the HPV infection (117).

1.8.2.2. Hormonal contraceptives use

Another co-factor associated with an increased risk of HPV-derived malignant lesions is the long-term use of hormonal contraceptives (118). Alterations in the hormonal mechanisms may deregulate HPV infection, leading to an increase risk of the integration status of the virus into the human genome (118). Studies have also found that estradiol, a component of hormonal contraceptives, may stimulate the transcription of the oncoproteins E6 and E7, leading to an increased risk for the development of cervical pre-cancerous lesions (119).

Additionally, estrogen and progesterone increase cell proliferation and in cooperation with the oncogenic HPV viral proteins, increase the risk of DNA damage particularly in the uterus transformation zone where those hormone receptors are particularly frequent (117).

1.8.2.3. Tobacco

Tobacco has been identified as a potent factor and co-factor for the development of several cancers (120). Women with persistent HPV infection that smoke continuously has one to three-fold increase in the risk of developing HPV-related malignancies when compared to non-smokers (121). Furthermore, it has also been reported an earlier onset of cervical cancer for patients presenting a smoking habit status (122). This may be explained by damages caused in the DNA by the chemical and carcinogenic components of cigarettes but also by the consequent decreased ability of the immune system to counteract viral infections (123).

1.8.2.4. HIV and *Chlamydia trachomatis*

HIV and other sexually transmitted agents like *Chlamydia trachomatis*, considered the most common transmitted bacterial infections, have also been identified as established co-factors that can promote the development of HPV-related malignancies, leading to immunocompromised states, with a decreased action of the host immune system against the virus (121,124).

1.8.2.5. Host genetic factors

Additionally, host factors like the genetic background and the immune system must be taken into consideration. A good immune system response will be activated in the presence of a pathogen, leading to an increased expression of phagocytes, cytokines and natural-killer cells (125). Taken this into account, an immunosuppressed person has an increased risk of an HPV persistent infection and consequently the development of malignant lesions (125). Several genetic polymorphisms have been identified and associated with an increased probability of developing HPV-related malignancies (126–128). One of the most studied has been the *p53* tumour suppressor gene rs1042522 (Arg72Pro) polymorphism with contradictory results (129,130). The presence of an arginine instead of a proline in the codon 72 of *p53* may lead to a stronger binding of the E6 to the p53 protein, which in its turn promotes an increment of its degradation (129).

1.9. HPV Epidemiology

1.9.1. Cervical Cancer

In 1842, the first association between cervical cancer and sexual activity was observed by Rigoni-Stern, which stated that prostitutes and married women had a higher incidence of cervical cancer when compared to nuns and virgin women. However, it was only after almost 150 years that Rigoni-Stern hypothesis was validated by the work of Harald Zur Hausen, in which HPV started being linked to the development of cervical cancer (4) (**Figure 1**). Nowadays, HPV is a well-established etiological factor for the development of cervical cancer and it is found in virtually 100% of those cancers, being approximately 84% of them SCC (131) (**Figure 10**). HPV usually infects the cervical transformation zone, a fragile metaplastic location between the endocervix and the ectocervix, which is not only the most sensitive oestrogen location but is also subjected to constant changes during the woman´s life and therefore it is believed to facilitate the establishment and the persistent of HPV infection and consequently HPV-induced lesions (132).

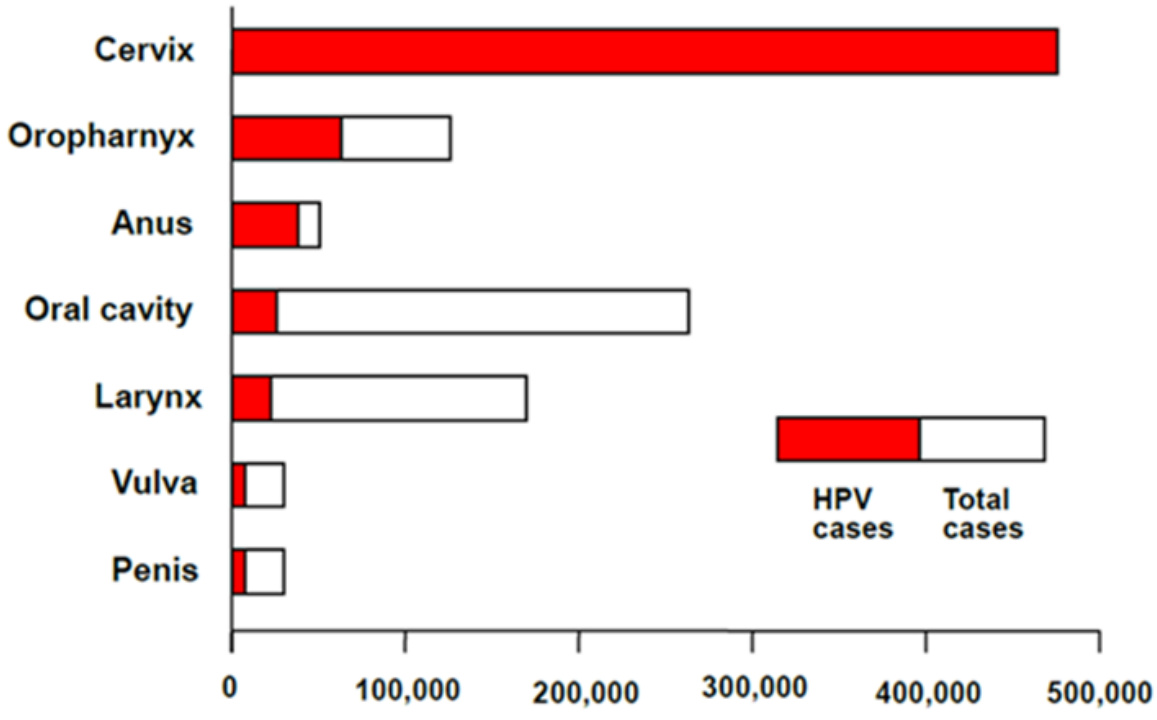


Figure 10. HPV-associated cancer cases in 2012, according to the International Agency for Research on Cancer (IARC).

83% of the HPV-related cancers are cervical cancers, the 4th worldwide most common cancer in females and the 2nd worldwide leading cause of cancer in women aged 15-44 (133). Even though the scenario seems negative, cervical cancer rates are decreasing in developed countries not only due to the increment of screening programs but also because of the implementation of prophylactic vaccination against HPV infection (134). However, HPV still remains a huge public health burden, especially in non-developed countries, where cervical cancer is still increasing at an alarming rate, accounting for 85% of cervical cancer worldwide (14).

Non-developed regions like Eastern Africa, Melanesia, Southern Africa and Middle and Western Africa have the higher incidence of cervical cancer (**Figure 11**). The highest mortality rates also belong to the previously referred regions, where HPV screening programs are almost inexistent. On the opposite hand, Western Asia, Australia/New Zealand, North America and Western Europe show the lowest incidence rates of cervical cancer (**Figure 11**). In Portugal, cervical cancer is the 10th most common cancer, where the most recent report shows 9.0 cases per 100.000 females, being the 23rd European country with higher cervical cancer rates (14).

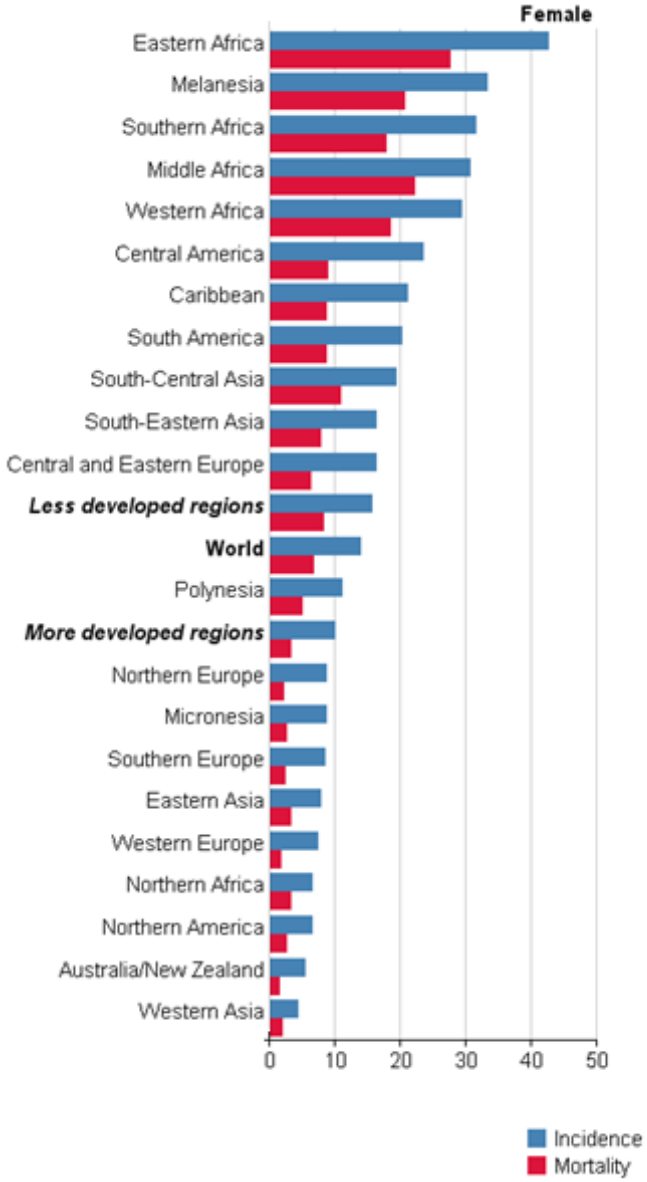


Figure 11. Worldwide Age-Standardized cervical cancer incidence and mortality per 100.000 females, IARC 2012.

1.9.2. HPV-induced anogenital cancers

Even though the knowledge of HPV role in the cervical carcinogenesis is well established, in anogenital cancers, englobing the vulvar, vaginal, anal and penile cancers it is still limited (135). While cervical cancer's incidence has been decreasing, anogenital HPV-induced cancers are increasing (14). According to the international agency for research on cancer (IARC), HPV is the worldwide annual cause of 8500 cases of vulvar cancer, 12000 cases of vaginal cancer, 35000 cases of anal cancer and 13000 cases of penile cancer (14) (**Figure 10**). The most common worldwide HPV-related anogenital cancers are anal and penile SCC. In anal carcinomas where 95% represent SCC, HPV is found in approximately 85% of the cases (**Figure 10**), particularly in the anal transformation zone between the rectum and the anus, being the most important factor for the anal carcinogenesis and the HPV16 the most prevalent HPV type (136). Women seem to be more susceptible to develop anal carcinoma, although HIV-infected men who have sex with men present the higher risk (137,138). In penile cancer, HPV is found in approximately 20-30% of the cases (**Figure 10**), being HPV16 also the most prevalent HPV type and SCC representing 95% of the total cases (139). However, even though penile HPV infection is common, the development of cancer is very rare. Other factors that can lead to the development of this carcinoma are lack of penile hygiene, smegma retention and phimosis (140). A distinct molecular pathway is observed between cancers derived from these factors or HPV infection, however, due to the fact that penile SCC is very rare, it is still much understudied (141). Both HPV-induced anal and penile cancers have particularly high-incidence in more developed regions like Northern Europe, North America, South America and Australia, possibly due to an increase of HPV transmission by sexual behaviour change (142) (**Figure 12**). In Portugal, in 2012, the age-standardized incidence rate (ASR) of HPV-positive anogenital cancers was 0.9 per 100.000 people.

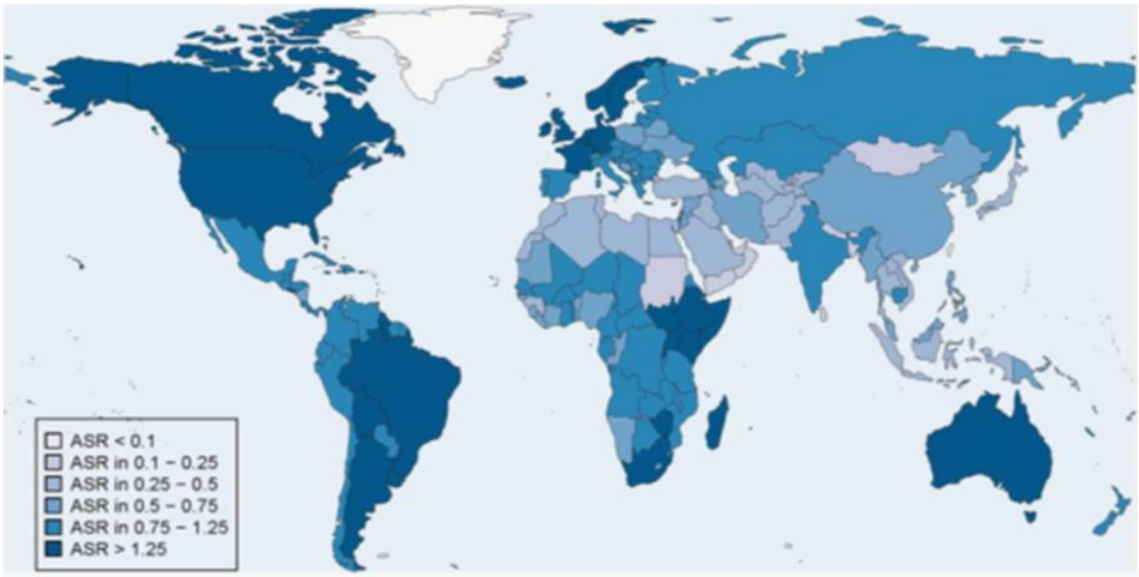


Figure 12. Worldwide age-standardized incidence rate (ASR) of anogenital HPV-positive cancer cases per 100.000 people, both sexes. IARC 2012.

1.9.3. HPV-induced head and neck cancers

Head and neck squamous cell carcinoma (HNSCC) is the fifth most common cancer worldwide, afflicting approximately 500.000 people annually, being 90% of the cancers SCC (143). Even though most of these cancers are associated with alcohol and tobacco consumption, infection by HPV has also been linked with their development, appearing in younger individuals that don´t smoke, drink or are immunosuppressed (144).

Depending on the anatomic location, HPV prevalence worldwide in HNSCC is approximately 20-40% (**Figure 10**) and reaching as high as 70-80% prevalence in the developed world (145). However, it is particularly high in the oropharyngeal anatomic location (≈40%), namely in the deep crypts of the palatine, lingual tonsils and base of the tongue compared with the larynx and the oral cavity, mainly due to different tissue architectural backgrounds and stromal microenvironments (146,147). In fact, the rate of HPV-induced oropharyngeal squamous cell carcinomas (OPSCC) is increasing so dramatically that in 20-25 years, HPV will be the main etiological factor related to the development of this carcinoma (148). Additionally, an increase of 225% of HPV-induced OPSCC from 1998 to 2004 has been noted compared with a regression of 50% of those cancers related to alcohol and tobacco consumption (148). However, even though oropharyngeal HPV-associated cancers are increasing so dramatically, there is still a huge gap in the knowledge of the carcinogenic cascade in this anatomic region when compared with the other HPV-cancers (**Table 2**)

Table 2. HPV-associated precancerous lesions and SSC

	Female		**Male**	
	Precancerous lesion	Cancer	Precancerous lesion	Cancer
Uterine cervix	CIN 3	SCC		
Vagina	VaIN3	SCC		
Vulva	VIN3	SCC		
Penis			PIN3	SCC
Anus	AIN3	SCC	AIN3	SCC
Oropharynx	?	SCC	?	SCC

Globally, per year, there are an estimated of 38.000 HNSCC cancers attributable to HPV and just like HPV-induced anogenital cancers, their incidence not only is increasing but is also higher in more developed regions namely North America and Europe (**Figure 13**). This fast-growing neoplasia affects 3 times more males than females from 35-55 years old explained by a higher probability of HPV transmission between vagina-oral mucosae rather than penile-oral mucosae, higher number of sexual partners by the males but also due to a stronger immunity response of the women that was probably previously infected and therefore immune to an HPV oral infection. This risk is even higher in women that have sex with women (149). HPV16 is found in more than 90% of the oropharyngeal cancers and it is not clearly understood why this HPV type has a much higher incidence, alongside with the anal carcinomas (85%), when compared with the uterine cervix where HPV16 is found in only 50% of the cases (150). Tissue microenvironment and specificity or viral preference can be the reason. In Portugal, in 2012, the ASR of HPV-positive head and neck cancers was 1 per 100.000 people.

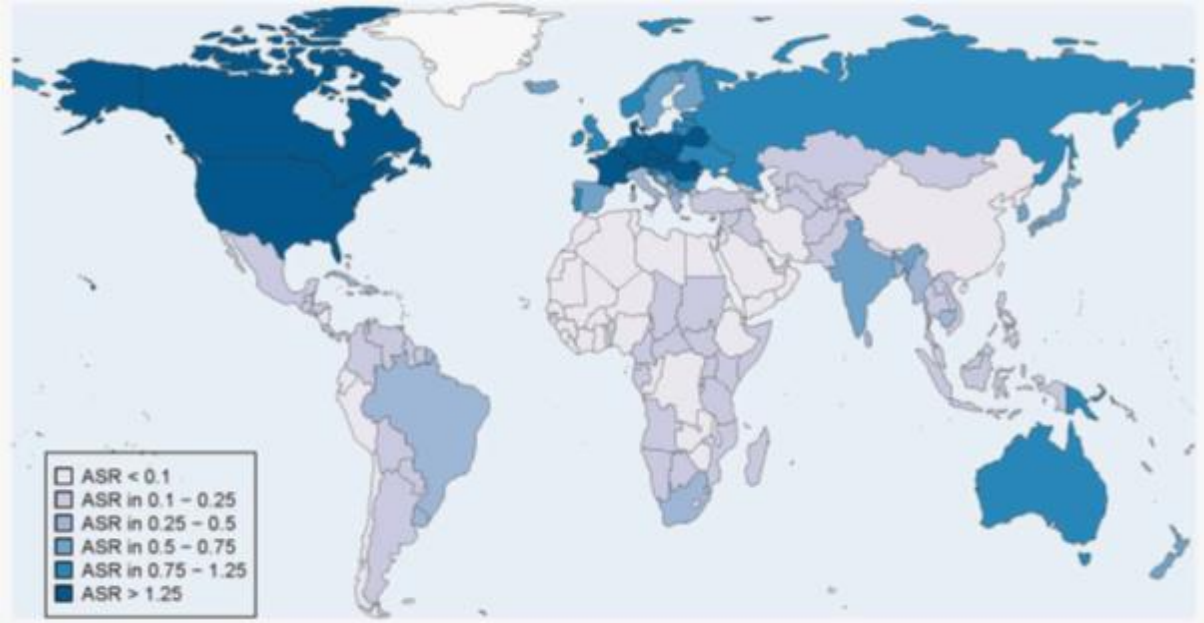


Figure 13. Worldwide ASR of head and neck HPV-positive cancer cases (oropharynx, oral cavity and larynx) per 100.000 people, both sexes. IARC 2012

1.10. K14-HPV16 mouse model

One of the biggest challenges in the study of the HPV life cycle and carcinogenesis is the inexistence of efficient *in vitro* protocols. As the virus is able to infect different anatomic sites, the influence of epithelial backgrounds, the tissue stromal microenvironment, the genetic expression and the immune system of the host may play an important role. Nowadays, the inexistence of *in vitro* approaches that mimic and recreate the three-dimension structure of the epithelium, which is crucial for virus infection and propagation, as well as animal models that characterize the HPV natural infection limits the understanding of the papillomavirus biology infection pathway. The K14-HPV16 mouse model appears to be one of the best approaches for the study of the carcinogenic cascade induced by HPV (151). The FVB/n inbreed mouse background used in this study, not only is characterized by a high reproductive performance and large litters but is also more permissive to SCC, when compared with other inbreed backgrounds like the *C57BL/6*, *BALB/c* and *SSIN/SENCAR* (152).

The K14-HPV16 model of induced neoplastic progression expresses the early genomic region of the high-risk HPV16 from the nucleotide 97 to the 6152, expressing E6, E7, E1, E2, E4 and E5 proteins (153) (**Figure 14**) .

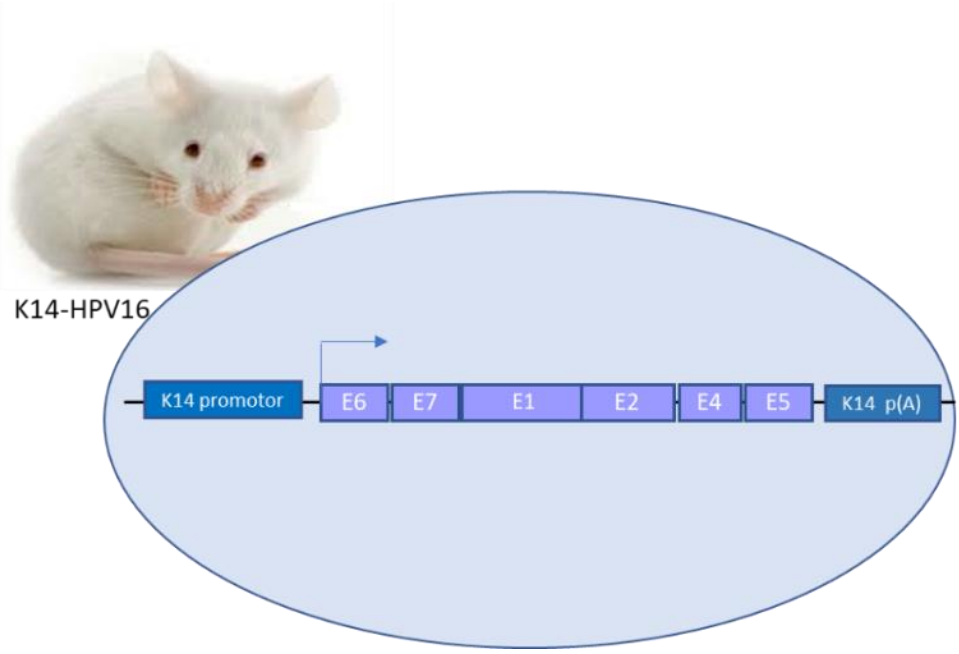


Figure 14. K14-HPV16 genomic construct.

The K14, one of the primary keratin proteins found in the basal membrane of stratified squamous epithelia is used as a promoter by directing the expression of the viral proteins to this cell type, not only mimicking the mechanism of infection but also the HPV-related lesions´ development by HPV integration into the host genome. The integration process happens in approximately 80% of the uterine cervix and HPV-induced anal carcinomas as well as 40% of HPV-induced oropharyngeal carcinoma while in other anogenital areas is not well documented (153–155). The transgene construct is also flanked downstream by a 500-base-pair polyadenylated signal, crucial for mRNA maturation mechanisms and protection against enzymatic degradation (153) (**Figure 14**).

It has been shown by different studies that the construct directed by the K14 promoter has a high-level expression in the different types of the squamous epithelia in transgenic mice, mimicking the unbalanced expression of the viral proteins in the onset of the human disease (156,157). Studies have also shown that the expression of the E6 and E7 oncoproteins is higher than the expression of E2 protein throughout all the epithelial layers, also mimicking what happens in humans after HPV integration (158). However, the total absence of E2, which usually happens after HPV integration in the human genome, doesn't occur in this mouse model, being one of the current limitations.

The K14-HPV16 mouse model allows the study of HPV-related lesions that develop at multiple epidermal and mucosal sites (152,153). Ear and chest skin are consistently affected by LSIL starting to appear phenotypically at four weeks old followed by HSIL as well as the development of anal papillomas (153). The K14-HPV16 mouse model mimics the human HPV-induced carcinogenesis once, the LSIL are histologically similar to the LGSL observed in humans, alongside, the HSIL lesions are similar to the HGSL in humans, the precursor lesion just before the development of carcinoma, that is characterized by an increased number of basaloid cells with irregular and hyperchromatic nucleus (159). The observed SSC in the K14-HPV16 mice and in HPV-related cancers in humans are also similar, with clusters of malignant epithelial cells surrounded by fibrovascular stroma (159). Studies also have shown that the immune activation and the consequent response against the expression of the viral oncoproteins in the K14-HPV16 mouse model lead to a chronic recruitment of inflammatory cells, which promote tumoral progression (160). Additionally, the K14-HPV16 is also characterized by cephalic alopecia together with an extensive hyperkeratosis and atrial erythema (**Figure 15**). Most of these lesions occur approximately at 20-30-week-old mice. A high mortality rate (> 50%) is observed after this period, while wild-type mice with FVB background usually live from 52 to 65 weeks.

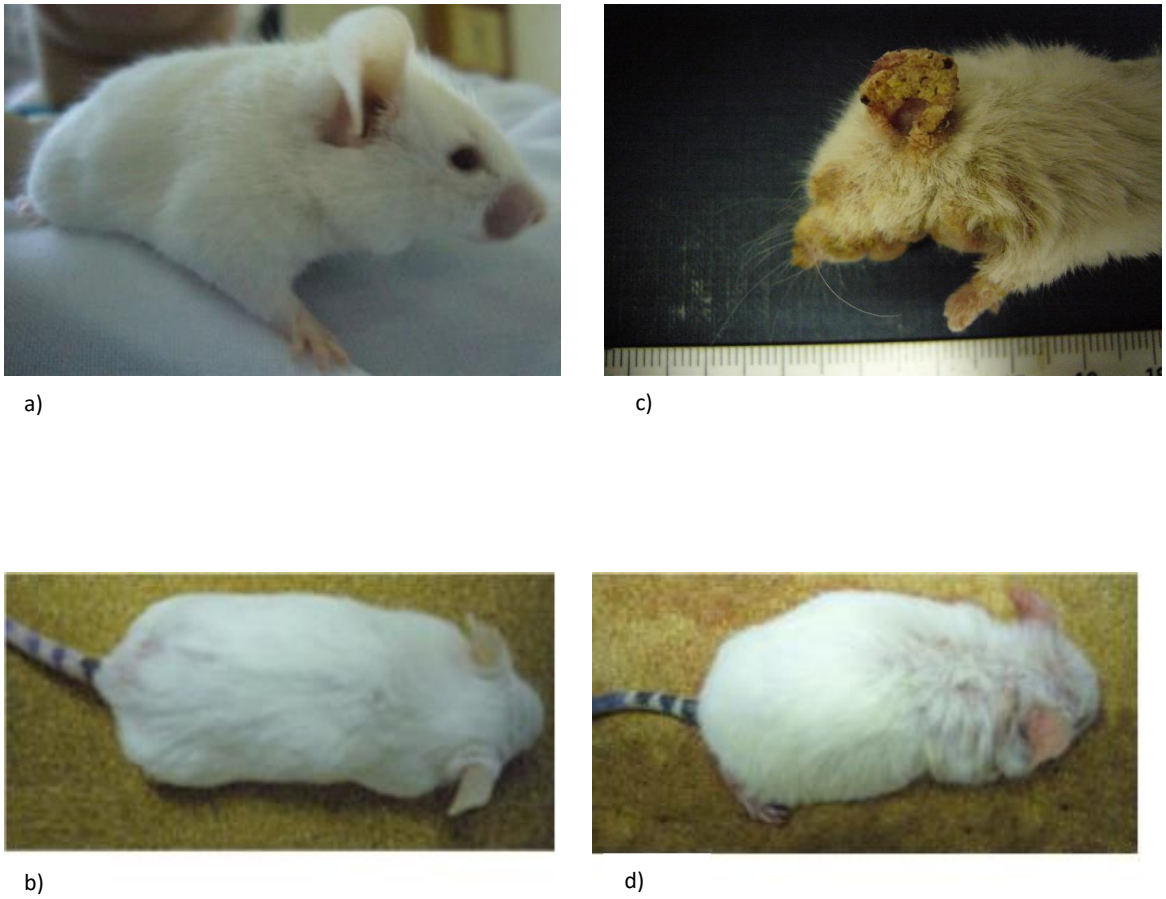


Figure 15. *Mus musculus. a) and b) Wild-type mice, 30 weeks-old; c) and d) transgenic mice K14 HPV16, 30 weeks - old.*

1.11. The Big Question:

The diverse stromal microenvironment, epithelial background, secretions, microbiota, genetic expression and hormonal responses of the reproductive, anogenital and oropharyngeal anatomic regions may influence the behaviour of the HPV viral proteins (147). All these variants may also influence the response of the host immune system.

Recent studies show that changes in the vaginal microbiota can increase the risk of HPV infection (161). On the other hand, an increase of HPV infection due to microbiota fluctuations in the anus and head and neck anatomic region still needs to be assessed. Another example of a different response in HPV-infected organs is the human α-defensin 5 (HD5), that can block HPV infection since it can prevent the furin-mediated cleavage of the L2 protein, a crucial step for the virus entry in the cell (162) (**Figure 7**). This anti-viral protein is actively expressed in the vagina and vulva epithelia, however, poorly expressed in the cervix transformation zone, making this region more prone to HPV infections and therefore to the development of lesions (163). Additionally, hormones like oestrogen and progesterone can influence not only the immune system but also the HPV oncoproteins, mediating the development of lesions (164,165).

Studies have shown that HPV may behave differently not only according to the gender it infects but also depending on the different anatomic locations in which the virus is present. For example, the oropharyngeal region is particularly prone to HPV infection in comparison with the nearby oral cavity and larynx anatomic locations due to its architecture and lymphoepithelial background characterized by a disruptive basal cell layer that facilitates HPV viral access to the basal stem keratinocytes (147). This anatomic region has an HPV viral load 80.000x higher than any other location in the head and neck anatomic regions (147).

Understating these differences is crucial to develop novel and precise therapies that can have a different impact in the different anatomic sites of infection. There are still important and basic questions that still need to be answered regarding HPV infection. Do the HPV oncoproteins trigger a distinct grade of lesions on the different anatomic sites of infection? How does the different tissue stromal microenvironment of the uterine cervix, anal and oral epithelia allied with the HPV oncoproteins modulate tumour progression?

Aims and scope

II. Aims and Scope

The main goal of this study was to observe the influence of E6, E7 and E5 oncoproteins mRNA expression as well as the different lesions development on multiple anatomic sites usually infected by HPV that are characterized by the influence of gender, different epithelia backgrounds, genetic expression and tissue stromal microenvironments.

The specific aims are to:

I. Assess the expression of E6, E7 and E5 mRNA in female K14-HPV16 organs that are known to be affected by HPV in humans, namely the base of the tongue, anus and uterine cervix and correlate it with the histopathological features.

II. Assess the expression of E6, E7 and E5 mRNA in male K14-HPV16 organs that are known to be affected by HPV in humans, namely the base of tongue, anus and penis and correlate it with the histopathological features.

III. Assess the expression of E6, E7 and E5 mRNA in K14-HPV16 mouse bladder and compare it with the expression of the other studied organs. Correlate the mRNA expression with the histopathological characteristics.

IV. Compare the expression of E6, E7 and E5 mRNA in K14-HPV16 male and female base of the tongue and anal samples.

V. Correlate lesion progression with E6, E7 and E5 mRNA expression

Materials and methods

III. Materials and methods

3.1. K14-HPV16 transgenic mice

The generation of the K14-HPV16 mice on an FVB/n background has been previously described by Arbeit and colleagues (153), comprising the entire early coding sequence of wild-type HPV16. The K14-HPV16 mice were kindly donated by Drs. Jeffery Arbeit and Douglas Hanahan, through the USA National Cancer Institute Mouse Repository. The animal experiments were approved by the University of Trás-os-Montes and Alto Douro Ethics Committee (10/2013) and the Portuguese Veterinary Directorate (0421(000/000/2014) and carried out at the University of Trás-os-Montes and Alto Douro. The K14-HPV16 and the wild-type mice were maintained and bred according to the Portuguese (Portaria 1005/92 dated October the 23rd) and European (EU Directive 2010/63/EU) legislation, with light-dark cycle (12h light / 12h dark), controlled temperature conditions between 20°-24°C, relative humidity (50 ± 10%), using corncob bedding. Food in form of pellets and water were provided *ad libitum.* Groups of four K14-HPV16 mice males and five K14-HPV16 mice female were maintained in a type II cage with length, width, height: 26,5x20,5x14,5 cm and with a floor space of approximately 545 cm^2.

3.2. Experimental design and sample collection

Ten female and ten male K14-HPV16 mice, with 30-weeks-old, were randomized and blindly chosen to be used in the experiment. Several of the most common HPV-infected anatomic locations were collected. Ten samples of chest skin, the base of the tongue, anus, uterine cervix and bladder were collected from female K14-HPV16. Additionally, ten samples of chest skin, the base of the tongue (only 9 samples were collected), anus and penis samples were collected from male K14-HPV16 (**Figure 16**). Matched samples were used for E6, E7 and E5 mRNA quantification and histological analysis. All mice were sacrificed under a combination of ketamine and xylazine anaesthesia followed by intracardiac puncture and exsanguination, according to the established protocols of the Center for the Research and Technology of Agro-Environmental and Biological Sciences in the University of Trás-os-Montes and Alto Douro. All samples were collected into TripleXtractor isolation reagent (Grisp®), macerated and kept at −80 °C until further use.

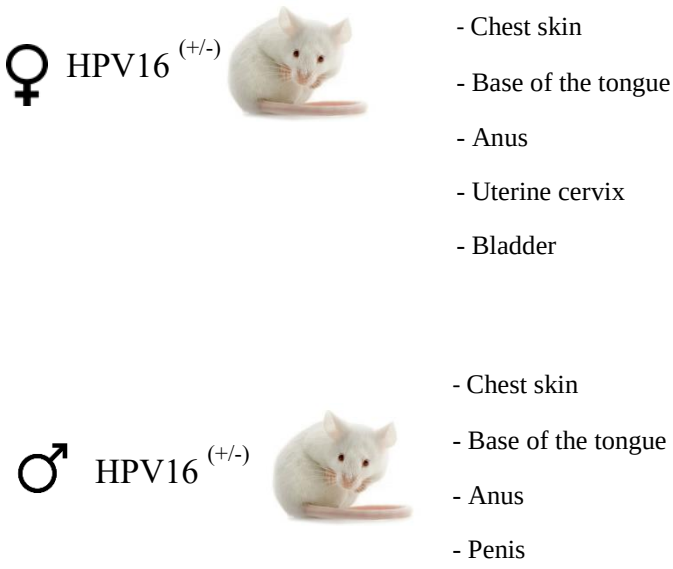


Figure 16. Samples collection of chest skin, anus, base of the tongue, uterine cervix, bladder and penis in K14-HPV16 mice female and male.

3.3. Mouse Genotyping

The K14-HPV16 mice were genotyped using a chest skin sample after organ collection. Wild-type mice were used as negative controls. DNA was extracted and purified using the GRS-Genomic DNA Kit Broadrange (Grisp®), according to the manufacturer's instructions. DNA concentration and purity were assessed by the *NanoDrop®spectrophotometer v3.7* (*Thermo Scientific, Wilmington* DE, EUA). The HPV16-E7 and the reference β-globin genes were amplified using polymerase chain reaction (PCR) in order to confirm the integration of HPV DNA into the mouse genome by specific primers (**Table 3**). The 50 µL HPV16-E7 PCR reaction included 1x DREAM Taq buffer including 20mM $MgCL_2$, 0,2 mM dNTP´s, 0,4 µM of each primer, 1U of DNA Taq polymerase and 200 ng/µL of genomic DNA. The amplification conditions were as follows: strand DNA denaturation at 95°C for three minutes, followed by 40 cycles at 94°C for 45 seconds, 55°C for 45 seconds, 72°C for one minute and the final extension at 72°C for five minutes. The expected band size of 157 base pairs (bp) was then confirmed by electrophorese gel agarose 3%. The 30 µL β-globin PCR reaction included 1x DREAM Taq buffer including 20mM $MgCL_2$, 0,2 mM dNTP´s, 0,5 µM of each primer, 1U of DNA Taq polymerase and 200 ng/µL of genomic DNA. The amplification conditions were as follows: strand DNA denaturation at 94°C for three minutes, followed by 35 cycles at 94°C for 30 seconds, 60°C for 45 seconds, 72°C for 90 seconds and the final extension at 72°C for ten minutes. The expected band size of 494 base pairs (bp) was then confirmed by electrophorese gel agarose 1,5%.

Table 3. HPV16-E7 genotyping primer sequence. F - forward, R - reverse.

Gene	Primer Sequence (5'->3')	Amplicon size (bp)
E7	F: GGAGGAGGATGAAATAGATGG R: GCCCATTAAGAGGTCTTCCAA	157
β-globin	F: CCAATCTGCTCACACAGGATAGAGAGGGCAGG R: CCTTGAGGCTGTCCAAGTGATTCAGGCCATCG	494

3.4. Reference genes selection

The genes used as references must fulfil several criteria. One of the most important principles is that their expression should not vary within the tissues or cells. Additionally, they should not be influenced by the experimental procedure (166). Our reference gene expression stability analysis was based on two steps. We first identified the likely candidates for our experimental procedure, followed by the analysis of the stability of the chosen candidate genes by real-time polymerase chain reaction (RT-PCR). Taking this into consideration, we chose three widely used reference genes, the TATA-box binding protein (*TBP*), the hypoxanthine guanine phosphoribosyl transferase (*HPRT*) and beta-2-microglobulin (*β2m)*, that showed to have the least variance within the different tissues in mice (167). Those three genes were then analysed by RT-PCR in the uterine cervix, the base of the tongue, anus, penis and bladder samples in order to choose the most stable reference gene to be used in subsequent tests. The stability value was assessed by the *NormFinder* statistical program (168). This program requires at least 8 samples per experimental group and a minimum of three reference genes. The software analyses the inter- and intra-group variability, ranking them by their stability values, in which a lower value corresponds to a more stable gene.

3.5. RNA extraction/purification and cDNA synthesis

Total ribonucleic acid (RNA) was extracted from the tissue samples using the High Pure RNA Isolation Kit (Roche®) and treated with *DNase* I to avoid genomic DNA contamination. First strand synthesis of mRNA, complementary DNA (cDNA) was carried out by using the High Capacity cDNA Reverse Transcriptase kit (Applied Biosystems®). The reverse transcriptase reaction contained a total volume of 20µL with a 10µL master mix containing 1X RT buffer, 1X dNTP mix 1X RT Random primers and 2,5 U of MultiScribe™ Reverse Transcriptase as well as 10µL of RNA sample.

The amplification conditions were as follows: 25°C for 10 minutes, 37°C for 120 minutes and a reverse transcriptase inactivation step at 85°C for five minutes. All reverse transcriptase reactions included a negative control. The reaction was performed in the Mycycler Thermal cycler (Bio-Rad).

3.6. RT-PCR

After cDNA synthesis, real-time PCR analysis was conducted using the Fast SYBR Green Master Mix (Applied Biosystems®) and performed on the StepOne qPCR Real-Time PCR device (Applied Biosystems®). The RT-PCR reaction contained a total volume of 10µL, of which 5µL of the fast SYBR GREEN master mix, a primer concentration of 0,2 mM, 1µL of the respective cDNA and dH_2O until the final volume. The cycling conditions were 95°C for 20 seconds, followed by 40 cycles at 95°C for three seconds and 60°C-66°C (depending on the primer annealing temperature) for 30 seconds. An additional 95°C for 15 seconds, 60°C for one minute and 95°C for 15 seconds was used to confirm the amplification specificity of each reaction. Additional "No Template Control" (NTC) was used to ensure the absence of genomic DNA contamination. All qPCRs were run in duplicates and the average standard deviation within all samples and respective duplicates was 0,24. The PCR efficiencies of the mRNA targets were between 94%-100% with the consequent slope variation between 3,28-3,48. E6, E7 and E5 mRNA expression levels were normalized to the average expression of the two most suitable reference genes, *TBP* and *HPRT*, using specific primers, as described in **Table 4** (169–171).

Table 4. RT-PCR Primer sequences of E6, E7, E5, TBP, HPRT and β2m. F - forward, R - reverse.

Gene	Primer sequence (5'->3')	Main regulatory function	Amplicon size (bp)
E6	F: GAGCGACCCAGAAAGTTACCAC R: ACCTCACGTCGCAGTAACTGTTG	HPV viral oncoprotein	107
E7	F: ACCGGACAGAGCCCATTACAA R: GTGCCCATTAACAGGTCTTCC	HPV viral oncoprotein	120
E5	F: CTTTGCTTTTGTGTGCTTTTGTGTG R: AAAGCGTGCATGTGTATGTATTAAA	HPV viral oncoprotein	192
TBP	F: CAAACCCAGAATTGTTCTCCTT R: ATGTGGTCTTCCTGAATCCCT	Transcription regulation of most genes	131
HPRT	F:TGAAGAGCTACTGTAATGATCAGTCAAC R: AGCAAGCTTGCAACCTTAACCA	Recycling of purines by the purine salvage pathway	187
β2m	F: GGTCTTTCTGGTGCTTGTCTCA R: GTTCGGCTTCCCATTCTCC	Component of the major histocompatibility complex class I	103

3.7. Western blot Analysis

The protein expression of E6 and E7 oncoproteins was evaluated by western blot. Proteins were isolated from the all mouse tissue samples using the TripleXtractor isolation reagent (Grisp®) and according to the manufacture instructions, containing protease inhibitors (Halt™ Protease & Phosphatase Inhibitor Cocktail; Thermo Scientific) at a specified concentration. Protein concentration was read using the DC Protein Assay (BIO-RAD) and then separated by SDS-PAGE (4-20% pre-cast gels) under reducing conditions. Proteins were then transferred by the Trans-Blot Turbo (BIO-RAD) onto PVDF membranes, blocked with 5% milk (BIO-RAD) in PBS-Tween 0.05% for one hour at room temperature. Primary monoclonal HPV16 E6/HPV18 E6, dilution 1:200 (Santa Cruz Biotechnology, sc-460); HPV16 E7, dilution 1:200 (Santa Cruz Biotechnology, sc-6981) and β-Actin, dilution 1:200 (Santa Cruz Biotechnology, sc-811178), were incubated for two hours at room temperature. After several washing steps, the membrane was incubated with secondary antibody m-IgGk BP-HRP, dilution 1:1000 (Santa Cruz Biotechnology, sc-516102) and revealed by chemiluminescence.

3.8. Histological features

Samples for histological analysis were fixed in 10% neutral buffered formalin. The bladder was distended and fixed *in situ*, as previously described, to avoid histological artefacts (172). The fixed tissues were then dehydrated through graded alcohols and xylene and embedded in paraffin using an automatic STP 120 processor. The samples were histologically classified as 1) normal, if no alterations were observed in the epithelium, 2) LSIL, if characterized by a higher number of basal epithelium layers, however, without nuclear alterations and deformities (113). 3) HSIL, if a higher degree of disorganization and undifferentiated cells that extend beyond the lower third of the epithelium is observed, alongside with the loss of morphology as well as abnormal mitotic figures, namely the generally larger nucleus with two to three metaphysical groups, denser chromatin and cell membrane more prominent and distorted and 4) SCC, if a complete loss of differentiation and disorganization in almost all the epithelium is observed alongside with the invasion of the basal membrane (113). The histopathological analysis was performed by an experienced pathologist, affiliated to the Molecular Oncology and Viral Pathology group, at the Portuguese Institute of Oncology (IPO-Porto) to the Center for the Research and Technology of Agro-Environmental and Biological Sciences, University of Trás-os-Montes and Alto Douro and to the Federal University of Maranhão, Brazil.

3.9. Statistical Analysis

Statistical analysis was performed using the IBM SPSS Statistics for Windows (Version 24.0). Kruskal-Wallis, Mann-Whitney and Livak methods were used to evaluate statistical differences in normalized relative expression of *E6, E7* and *E5* genes among the different tissue samples (base of the tongue, uterine cervix, anus, penis and bladder). The incidence of histological differences in the different studied groups was performed using the chi-squared test. Results were considered statistically significant when *p* values were lower than 0.05.

Results

IV. Results

4.1. Mouse Genotyping

The presence of the HPV16 construct was confirmed by the amplification of the *E7* genomic region (157 bp amplicon) in the K14-HPV16 males and females used in this study (**Figure 17**). In the wild-type mice, used as negative controls, the HPV16 *E7* amplicon was not observed. In both K14-HPV16 and wild-type mice the *β-globin* gene was amplified. Phenotypically, while the wild-type mice did not show any epithelial lesions, all the mice in which the HPV16 was integrated into their genome presented several degrees of lesions in the chest skin and ear.

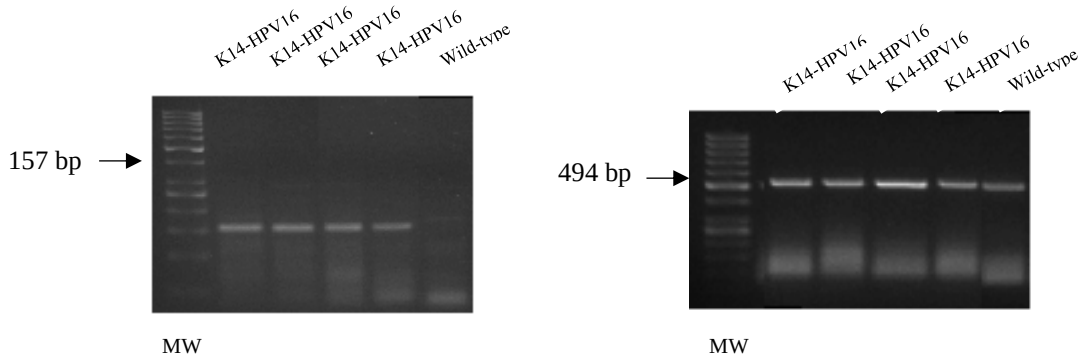


Figure 17. K14-HPV16 and wild-type mice genotyping. The presence of the HPV16 DNA was assessed by amplifying the E7 regions concomitant with the β-globin reference gene expression. The expected 157 bp band can be observed in the K14-HPV16 mice while the expected 494 bp band can be observed in both K14-HPV16 and wild-type mice. 3% and 1,5% agarose gel respectively, 50 bp gene ladder. MW – Molecular Weight

4.2. Reference genes selection

The stability values of the *TBP*, *HPRT* and the *β2m* reference genes were assessed by RT-PCR, using the Norm Finder statistical program (168). This program uses a mathematical algorithm in order to identify the best reference gene based on a pairwise variation analysis, according to the intra- and inter-group variation. Lower values of average $M^{NormFinder}$ indicate a higher stability expression. The stability values of the candidate genes were: *β2m*= 0,278; *TBP*= 0,437 and *HPRT*= 0,477 (**Figure 18**). However, the best combination of endogenous control genes is a combination of the average expression of *TBP* and *HPRT,* with a stability value of 0,221. The combined expression of *TBP* and *HPRT* was consequently used as reference genes in this study. The stability values of the candidate genes were similar with the ones presented in previous studies (167).

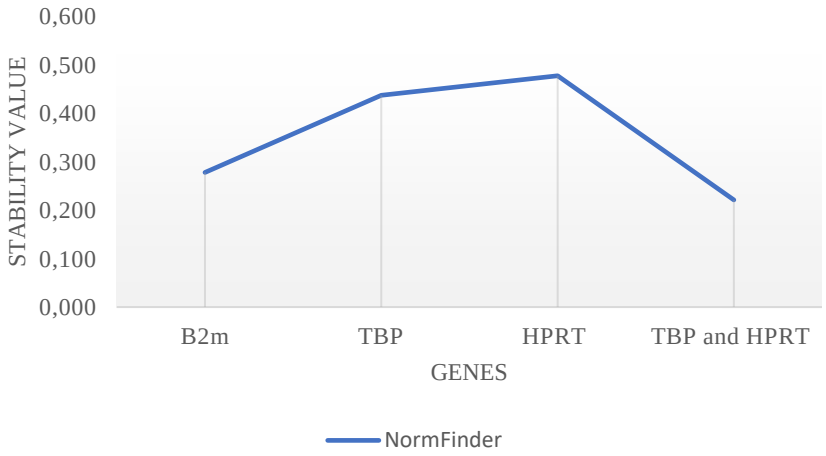


Figure 18. Normfinder analysis of the β2m, TBP, HPRT stability values.

4.3. Aim I

4.3.1. E6, E7 and E5 mRNA expression in K14-HPV16 female mice base of the tongue, anus and uterine cervix

Being the uterine cervix, the base of the tongue and the anus the most affected anatomic sites by HPV in women, we compared the E6, E7 and E5 oncogenic mRNA expression in the K14-HPV16 female mice tissue samples (**Figure 19**). The expression of the E6 oncogenic mRNA was similar between the base of the tongue, anus and uterine cervix samples (p=0,155). Additionally, the expression of the E7 and E5 mRNAs showed also no significant statistical difference between the base of the tongue and anal samples (p=0,199 and p=0,386, respectively). However, the uterine cervix was the anatomic location where more differences were found. The expression of the uterine cervix E7 mRNA was lower compared with the E7 mRNA expression in the base of the tongue and anal samples (p=0,016 and p=0,017 respectively). Alongside, the uterine cervix E5 mRNA expression was also lower when compared with the E5 mRNA expression of the anal samples (p=0,007) but with no statistically difference with the E5 mRNA expression in the base of the tongue (p=0,110). We can also observe a different expression pattern of E6, E7 and E5 mRNAs in the three, being the E6 mRNA the most expressed one followed by E7 and E5 (E6>E7>E5), (p<0,001).

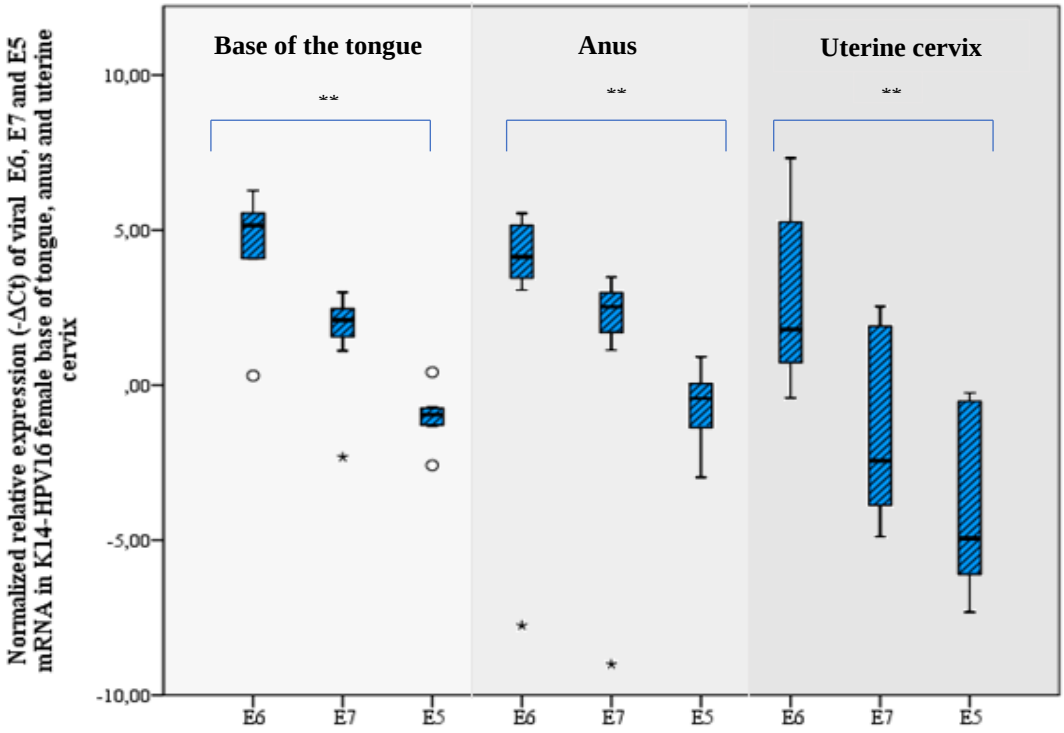


Figure19. Normalized relative expression (-ΔCt) of E6, E7 and E5 in K14-HPV16 female mice base of tongue, anus and uterine cervix.

** p<0.01, n=10; o = Mild outlier; * = Extreme Outlier

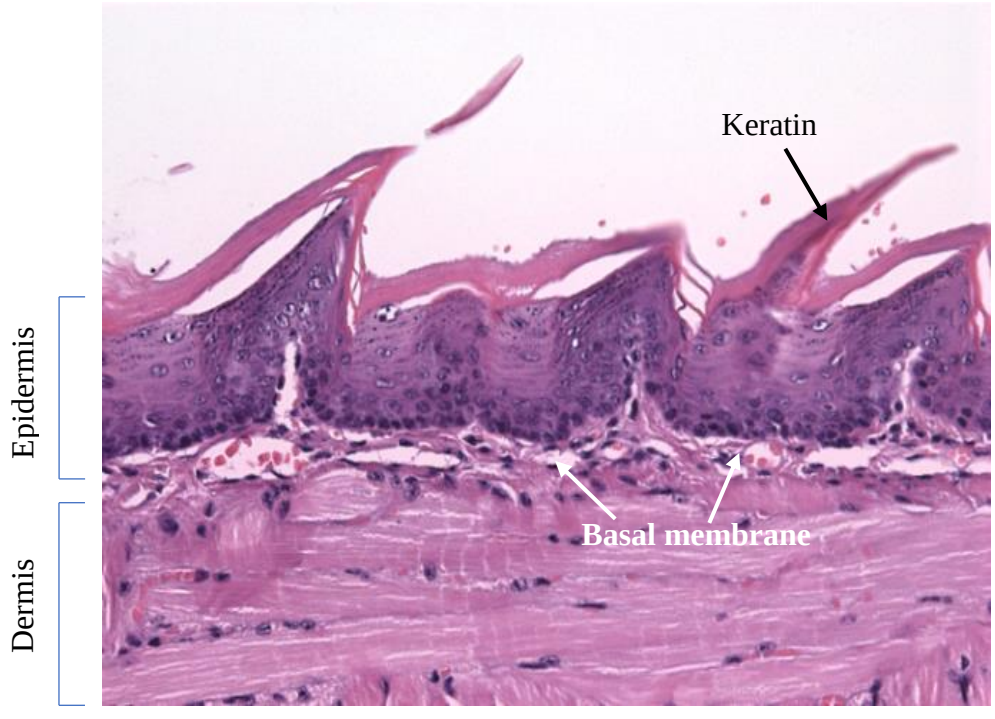
4.3.2. Histopathology of K14-HPV16 female mice base of the tongue, anus and uterine cervix

Regarding the histopathological features, 100% (10/10) of the uterine cervix samples showed normal epithelium with no associated lesions. Concerning, the anal samples, 10% (1/10) were classified also as normal epithelium without any notorious cellular alterations but the remaining 90% (9/10) developed LSIL. In the base of the tongue samples, 60% (6/10) were also classified as LSIL but 40% (4/10) of the samples developed more advance lesions, namely 20% (2/10) being classified as HSIL and other 20% (2/10) invasive SCC. The penetrance of the female base of the tongue, anus and uterine cervix lesions are described in **Table 5**.

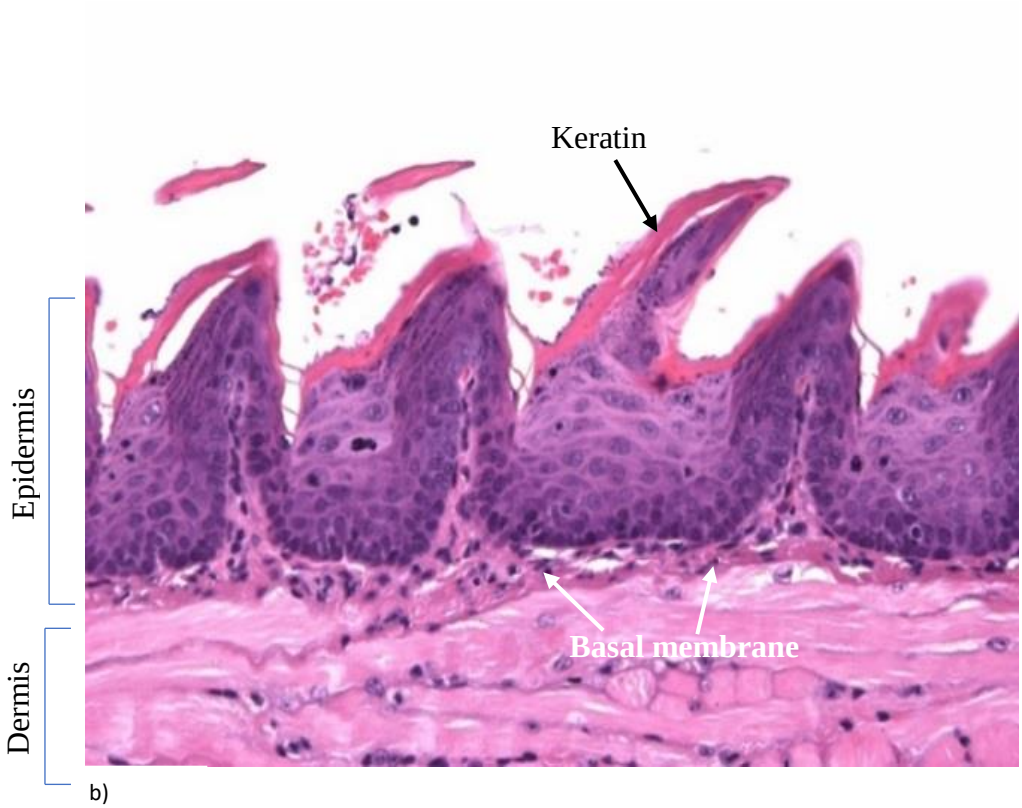
Table 5. Spectrum of HPV-induced lesions in K14-HPV16 females' base of the tongue, anus and uterine cervix.

	Age (weeks)	Organs	Normal	LSIL	HSIL	Invasive SCC
Female	30	Base of the tongue	----	60% 6/10	20% 2/10	20% 2/10
	30	Anus	10% 1/10	90% 9/10	----	----
	30	Uterine cervix	100% 10/10	----	----	----

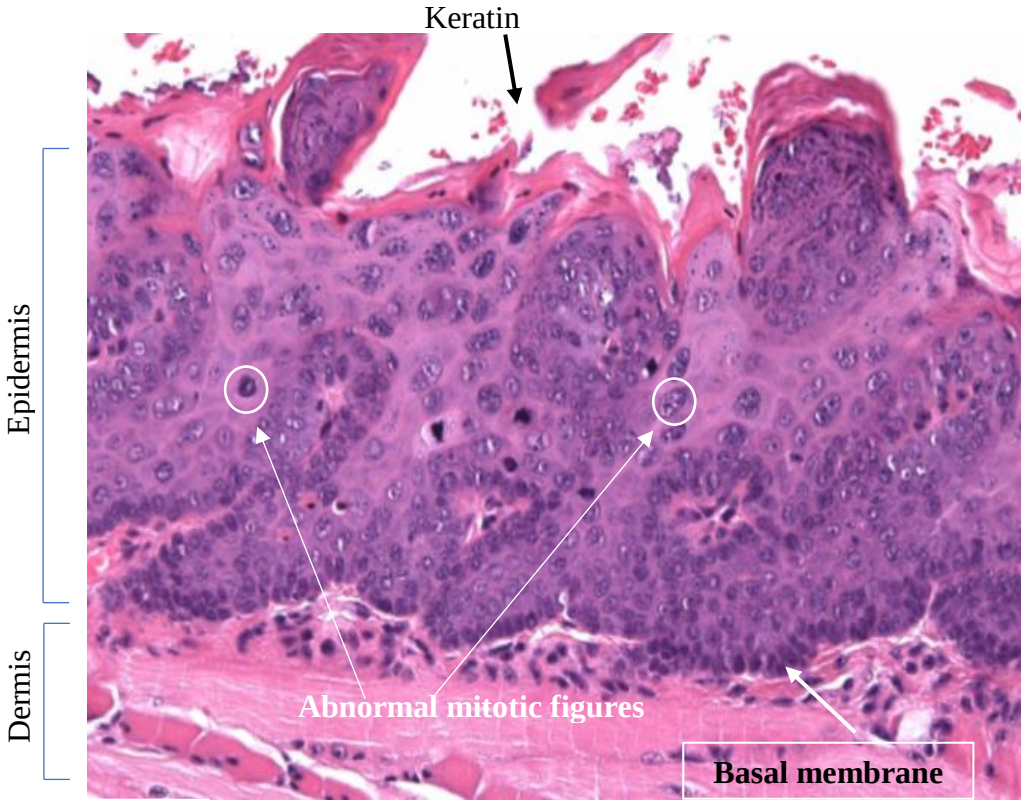
Images of the base of tongue normal tissue (**Figure 20 a**), LSIL (**Figure 20 b**), HSIL (**Figure 20 c**) and SCC (**Figure 20 d**), are displayed below.



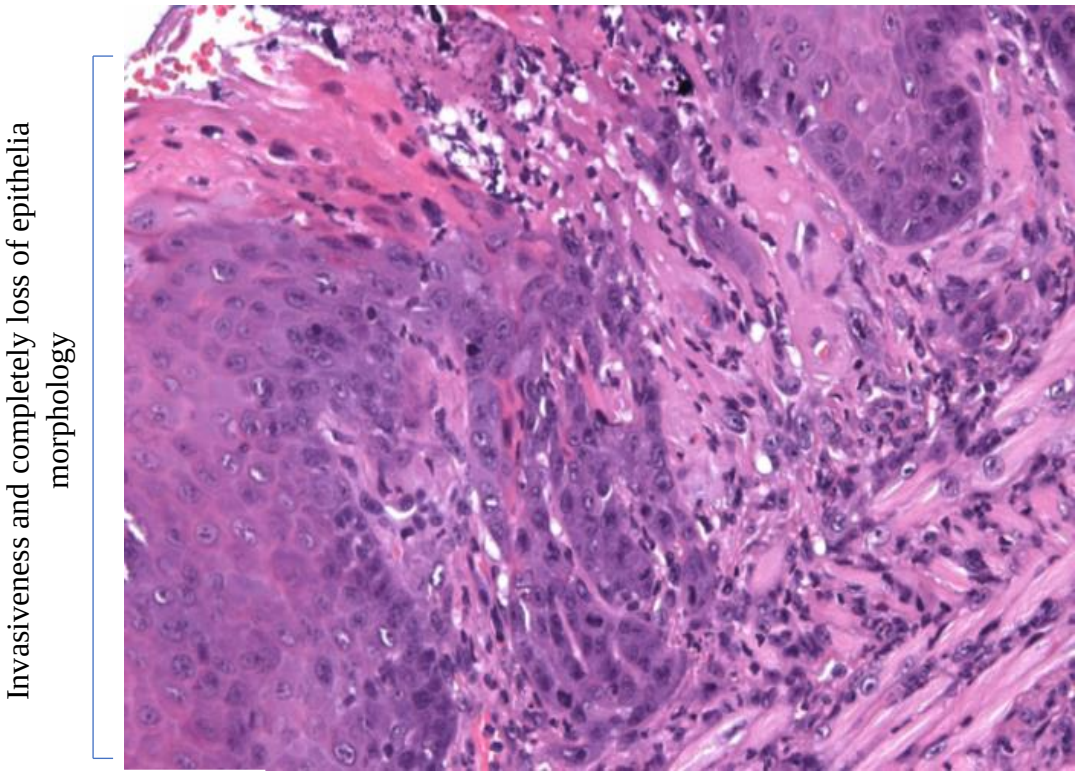
a)


Keratin
Epidermis
Dermis
Basal membrane

b)


Keratin
Epidermis
Dermis
Abnormal mitotic figures
Basal membrane

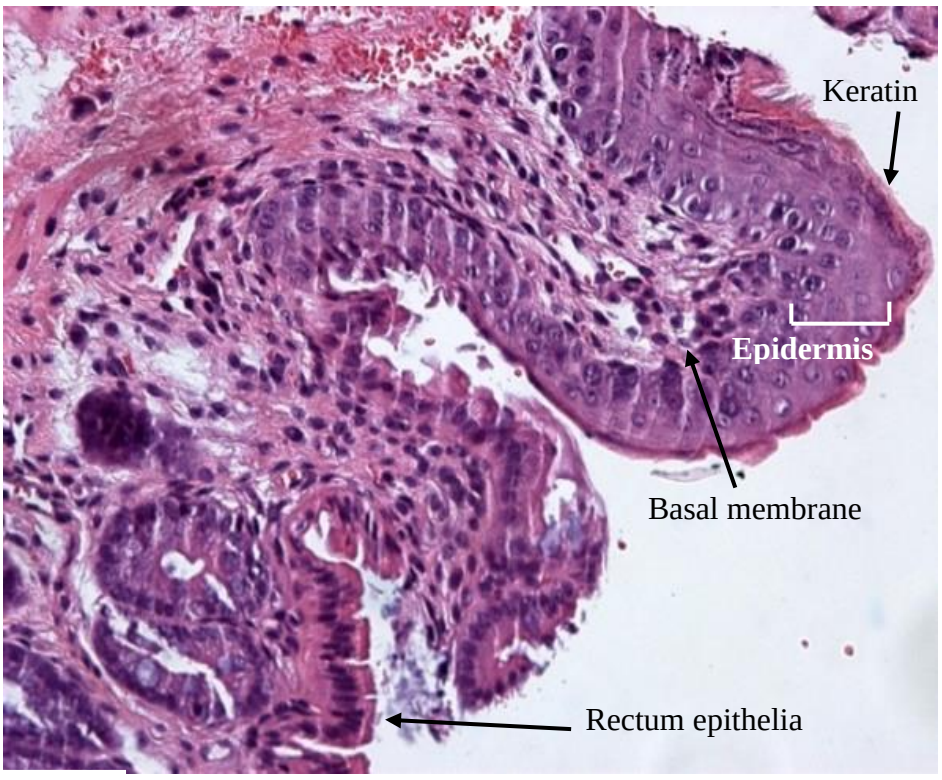
c)



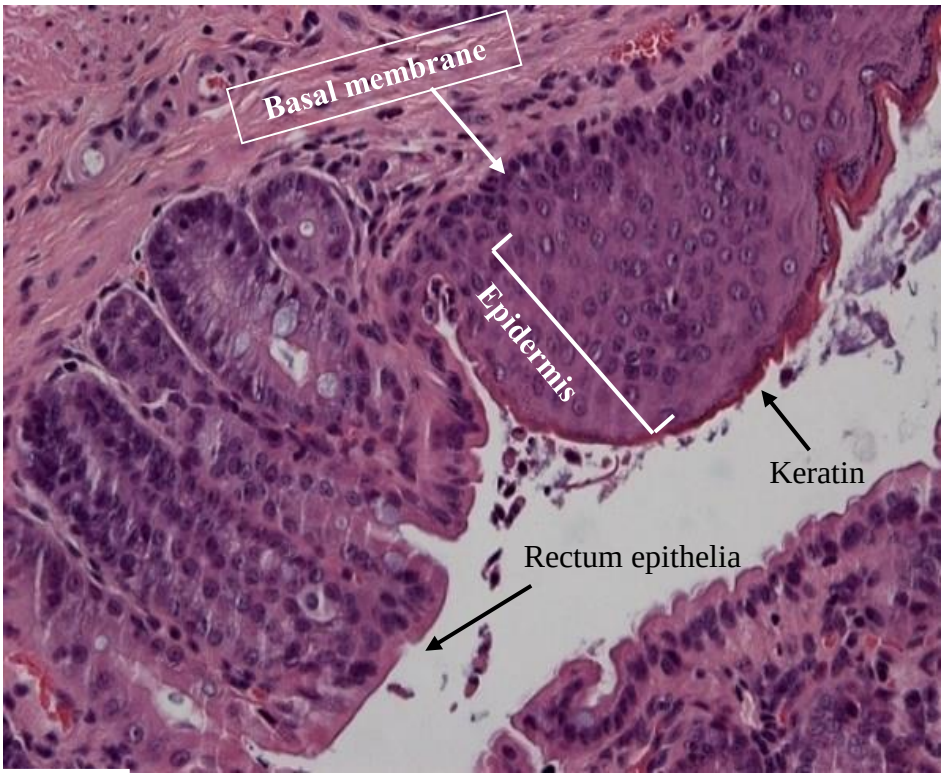
d)

Figure 20). Histological analysis of mice wild-type and K14-HPV16 base of the tongue samples. Hematoxylin and eosin stain (H&E), 200x. a) wild-type mice, showing normal base of the tongue epithelia, b) K14-HPV16 mice with LSIL in the base of the tongue, c) K14-HPV16 mice with base of the tongue HSIL epithelia and d) K14-HPV16 mice with base of the tongue invasive SCC.

Furthermore, images of the anal normal tissue (**Figure 21 a**) and LSIL (**Figure 21 b**) are also displayed in the following figures.



a)



b)

Figure 21. Histological analysis of wild-type and K14-HPV16 mice anal samples H&E, 200x. a) Wild-type mice, showing normal anal epithelia. b) K14-HPV16 mice with anal LSIL.

Additionally, an image of the uterine cervix normal epithelia is also displayed below (**Figure 22**)

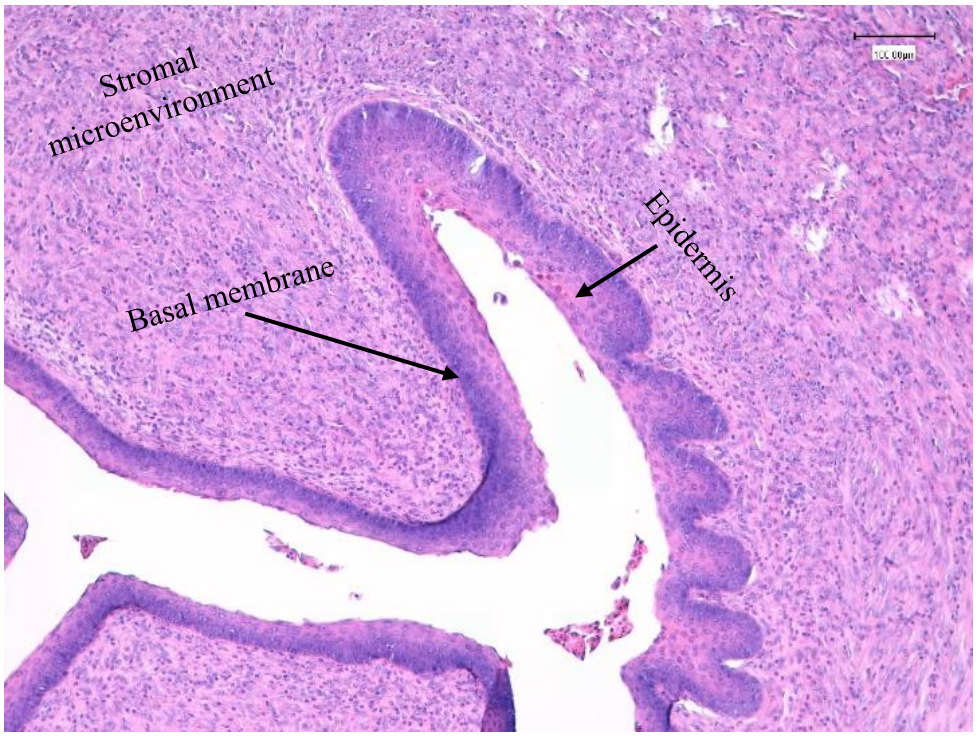
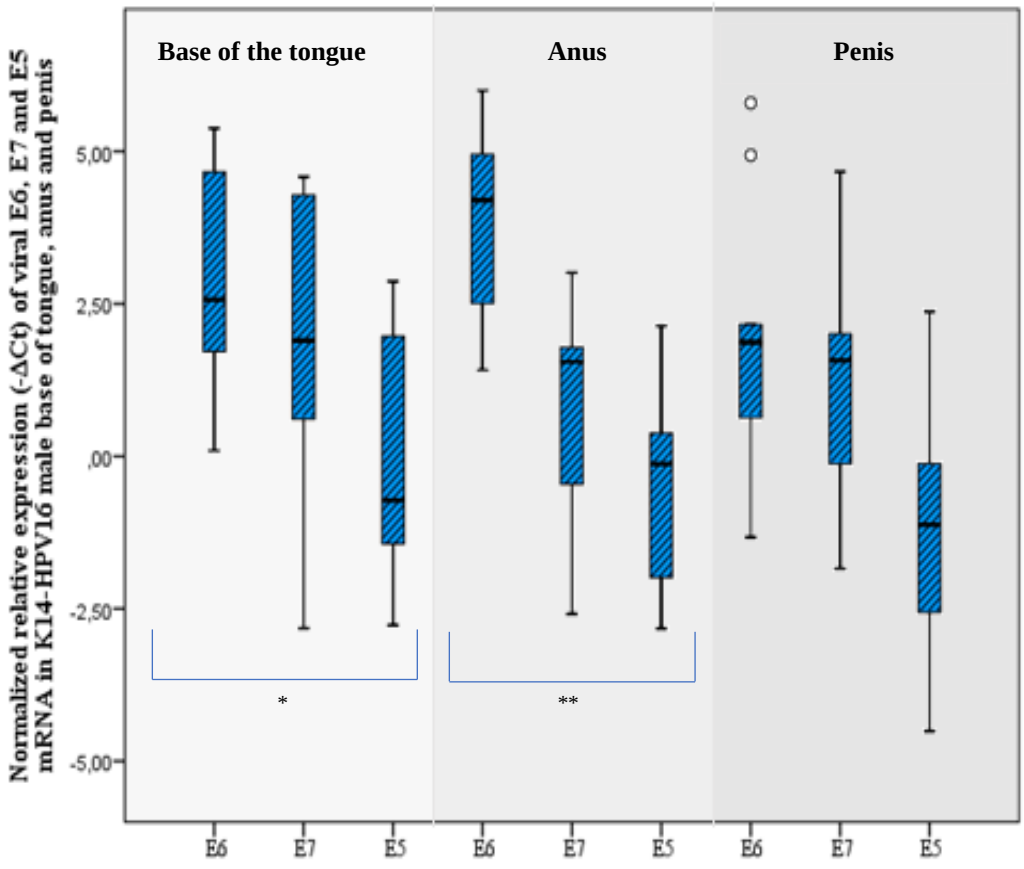


Figure 22. Histological analysis of K14-HPV16 mice uterine cervix samples, showing normal epithelium. H&E, 100x

4.4. Aim II

4.4.1. E6, E7 and E5 mRNA expression in K14-HPV16 male mice base of the tongue, anus and penis

We then quantified E6, E7 and E5 mRNA expression in the K14-HPV16 male base of the tongue, anus and penis samples, the most common HPV-infected anatomic locations in males. Even though the mRNA expression of the three mRNA oncoproteins showed to be less precise when compared with the female samples, the base of the tongue, anus and penis showed similar expression of E6, E7 and E5 mRNA, with no statistically differences observed (p=0,125; p=0,547; p=0,490, respectively) (**Figure 23**). Different expression patterns of E6, E7 and E5 mRNAs are also observed more evidently in the K14-HPV16 base of the tongue (p=0,047) and anus (p=0,001), in comparison with the penile samples (p=0,290).



* p<0.05, n=10

** p<0.01, n=10

o = Mild outlier

Figure 23. Normalized relative expression (-ΔCt) of E6, E7 and E5 in K14-HPV16 male mice base of tongue, anus and penis.

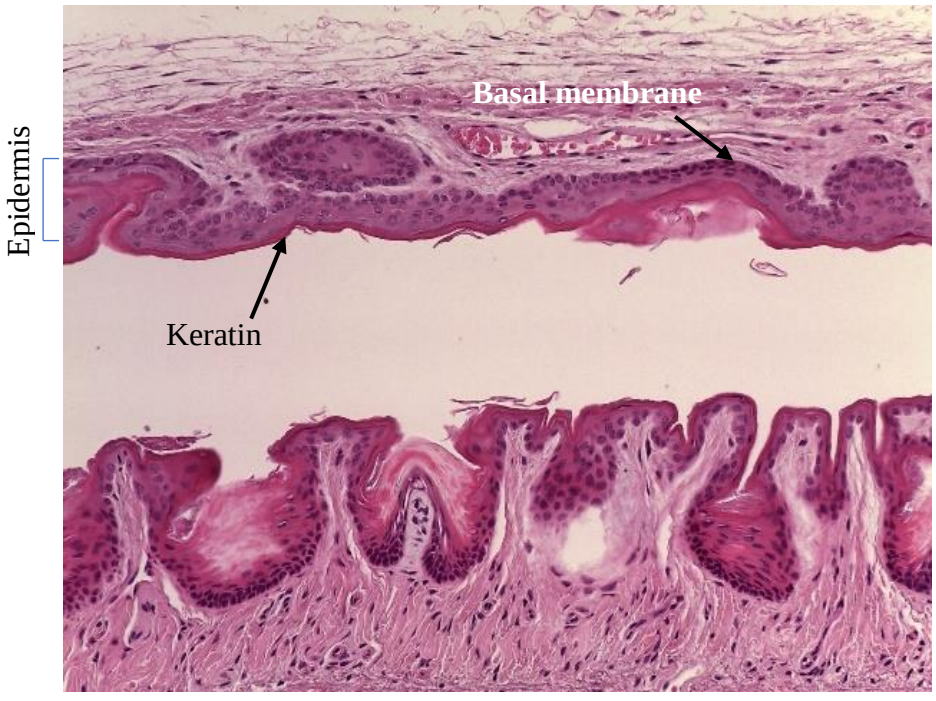
4.4.2. Histopathology of K14-HPV16 male mice base of the tongue, anus and penis

Histologically and similarly with the female samples, the K14-HPV16 male base of the tongue developed the most advanced lesions. 11% (1/9) of the base of the tongue samples were classified as normal epithelium, 55% (5/9) developed LSIL, 22% (2/9) developed HSIL and 11% (1/9) developed invasive SCC. Regarding the male K14-HPV16 anal samples, 100% (10/10) of the lesions were LSIL. Additionally, the most diagnosed lesion in penile samples was LSIL (80%) (8/10), whereas 20% (2/10) developed HSIL. The penetrance of lesions of the K14-HPV16 male mice base of the tongue, anus and penis is described in **Table 6**.

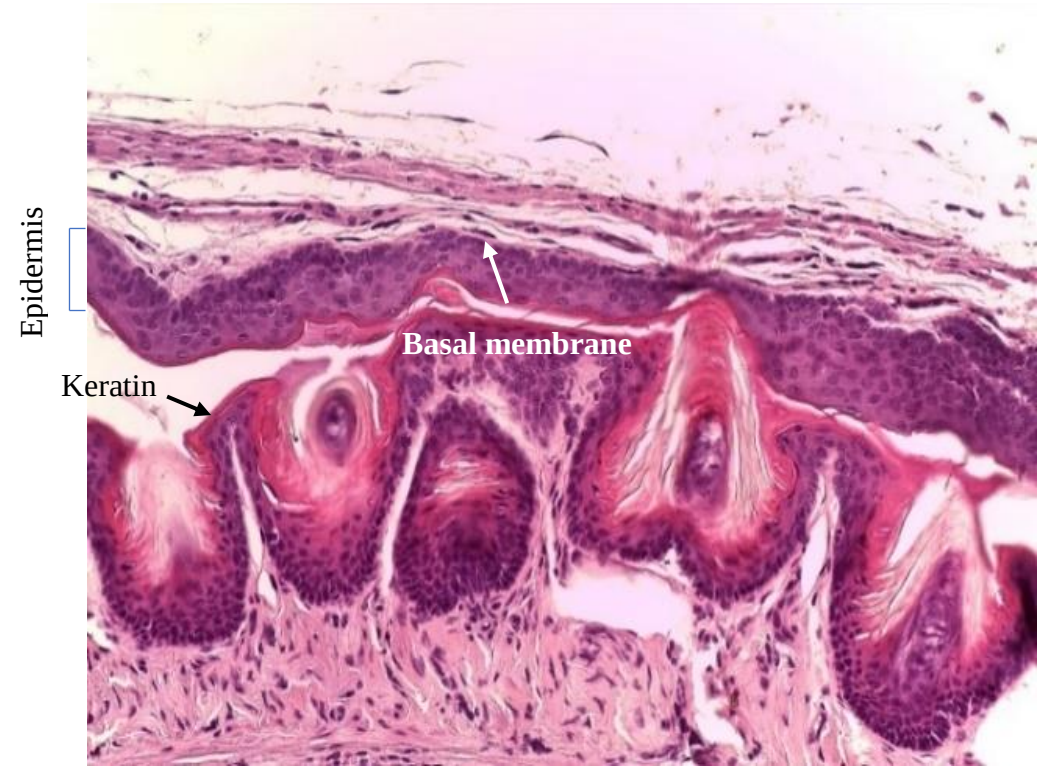
Table 6. Spectrum of HPV-induced lesions in K14-HPV16 males' base of the tongue, anus and penis.

	Age (Weeks)	Organs	Normal	LSIL	HSIL	Invasive SCC
Male	30	Base of the tongue	----	66% 6/9	22% 2/9	11% 1/9
	30	Anus	----	100% 10/10	----	----
	30	Penis	----	80% 8/10	20% 2/10	----

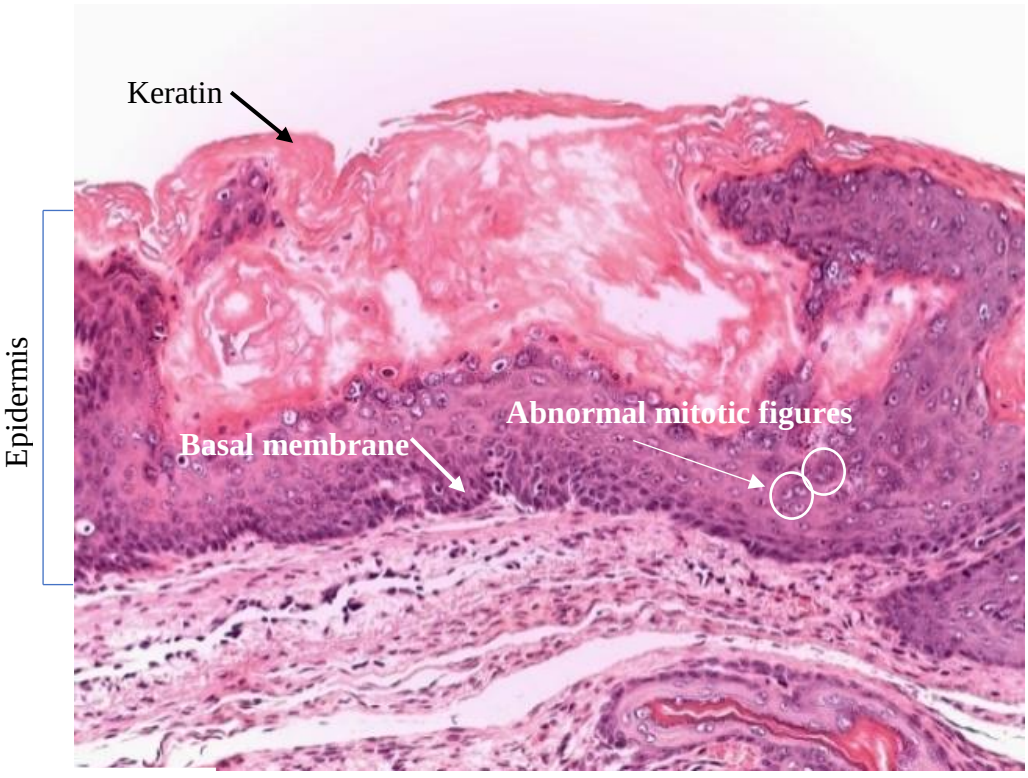
Images of the normal penile tissue (**Figure 24 a**), penile LSIL epithelia (**Figure 24 b**) and penile HSIL epithelia (**Figure 24 c**), are shown in the figures below.



a)



b)



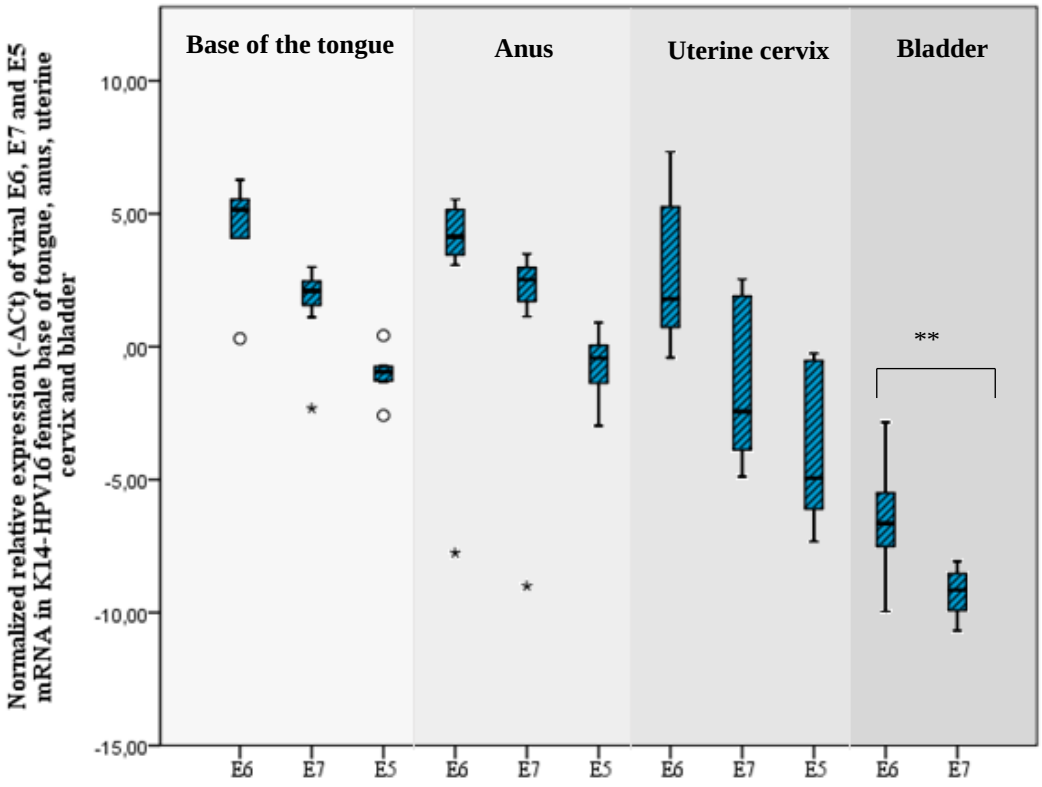
c)

Figure 24. Histological analysis of wild-type and K14-HPV16 mice penile samples H&E, 200x. a) Wild-type mice, showing normal penis epithelia, b) K14-HPV16 mice with penis LSIL. c) K14-HPV16 mice with penis epithelial HSIL

4.5. Aim III

4.5.1. E6, E7 and E5 mRNA expression in K14-HPV16 mice bladder

Posteriorly, we measured the levels of E6, E7 and E5 in the bladder samples (**Figure 25**). Due to the controversial role of HPV in bladder carcinogenesis, we have performed this analysis in the samples from one gender only, namely in the female mice and compared its expression with the samples from the base of tongue, anus and uterine cervix. Future studies, englobing the bladder of the K14-HPV16 male mice, will be performed. We found that the E6 and E7 transgenic expression was very low, almost residual when comparing with the oncogenic mRNA expression from the base of the tongue, anus and uterine cervix ($p<0,01$) (**Figure 25**). In bladder tissues, we did not detect E6 and E7 mRNA expression in four and five samples respectively. Alongside, no detectable E5 mRNA expression was observed in all the bladder samples. In the bladder, as similar as in other organs, there is also a different pattern expression between E6 and E7 mRNA expression ($p=0,045$).



** p<0.05, n=10, o = Mild outlier, *= Extreme outlier

Figure 25. Normalized relative expression (-ΔCt) of E6, E7 and E5 in K14-HPV16 female base of tongue, anus and bladder

4.5.2. Histopathology of K14-HPV16 mice bladder

Histologically, all K14-HPV16 bladder tissue was classified as normal, with no notorious cellular alterations (**Figure 26**). The penetrance of bladder lesions is described in **Table 7**

Table 7. Spectrum of HPV-induced lesions in the K14-HPV16 bladder samples.

	Age (Weeks)	Organs	Normal	LSIL	HSIL	Carcinoma in situ
Female	30	bladder	10/10 100%	----	----	----

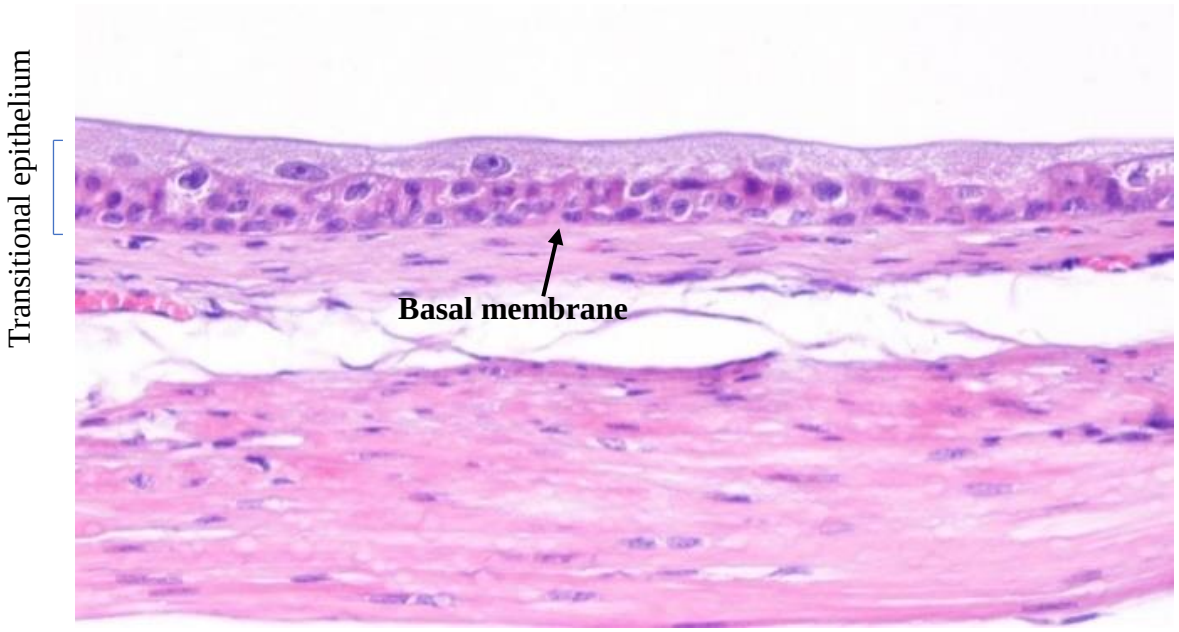
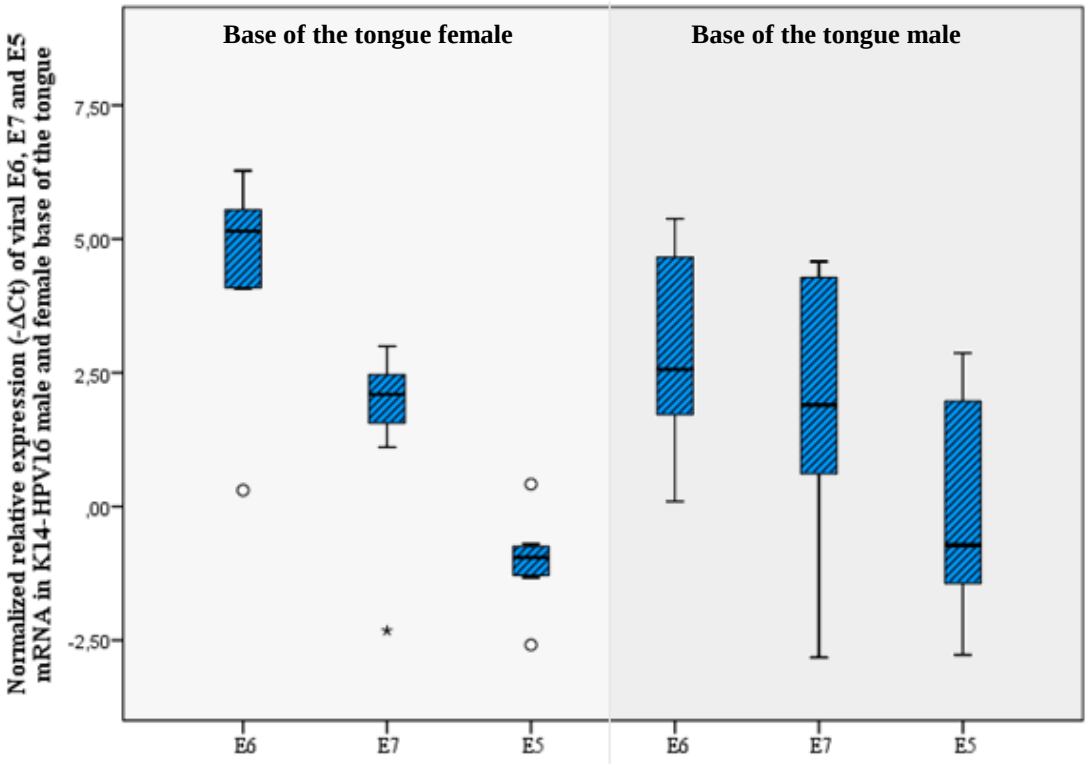


Figure 26. Histological analysis of K14-HPV16 mice bladder samples H&E, 200x. Normal bladder epithelia.

4.6. Aim IV

4.6.1. E6, E7 and E5 mRNA expression in K14-HPV16 male and female base of the tongue

The next step was to observe if there was a difference in the E6, E7 and E5 mRNA expression between genders, taking into consideration possible variations in physiological, immune and hormonal backgrounds. We observed a higher E6 mRNA transgene expression, namely six times higher, in the female base of the tongue when compared with the males' base of the tongue (p=0,027). However, E7 and E5 mRNA expression were similar between genders (p=0,806 and p=0,441 respectively) (**Figure 27**).



o = Mild outlier

*= Extreme outlier

Figure 27. Normalized relative expression (-ΔCt) of E6, E7 and E5 in K14-HPV16 female and male base of the tongue.

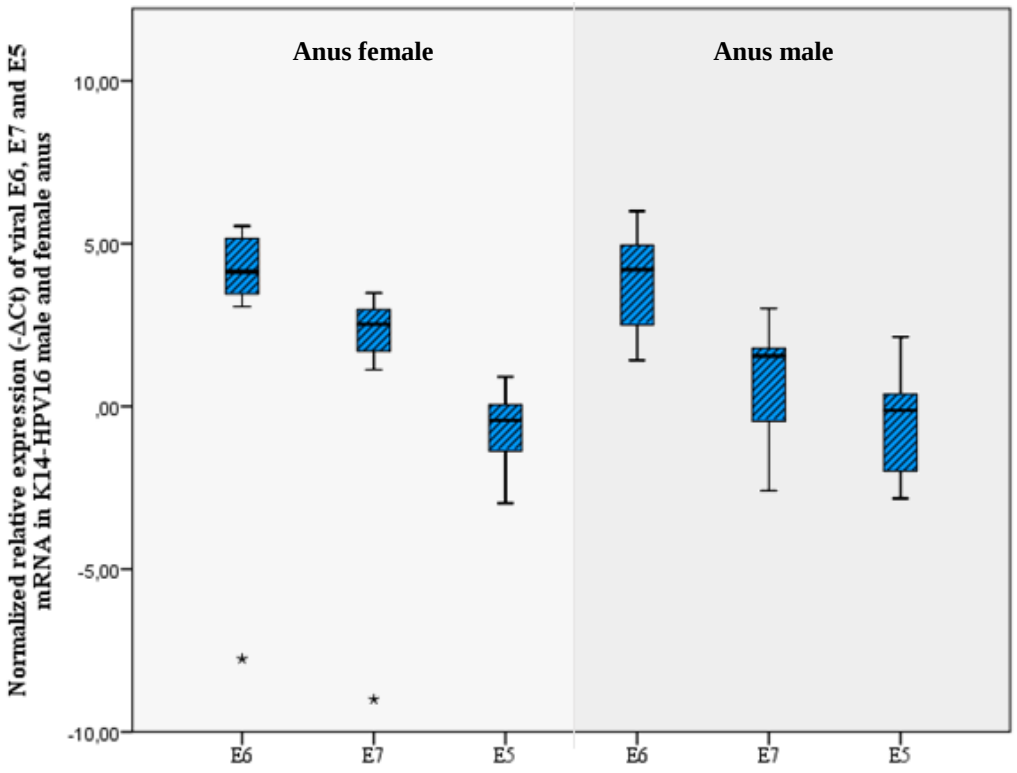
Histologically, the K14-HPV16 female mice base of tongue developed approximately 50% more invasive SCC when compared with the K14-HPV16 male mice base of the tongue (20% *versus* 11%). The penetrance of HPV-induced lesions in the K14-HPV16 base of the tongue male and female lesions is described in **Table 8.**

Table 8. Spectrum of HPV-induced lesions in the K14-HPV16 base of the tongue male and female.

	Age (Weeks)	Organs	Normal	LSIL	HSIL	Invasive SCC
Female	30	Base of the tongue	----	66% 6/9	22% 2/9	11% 1/9
Male	30	Base of the tongue	----	60% 6/10	20% 2/10	20% 2/10

4.6.2. E6, E7 and E5 mRNA expression in K14-HPV16 male and female anus

We also compared the E6, E7 and E5 mRNA expression between the K14-HPV16 female and male mice anal samples. We found that all the three transgene mRNAs were expressed similarly in both genders, with no statistically significant differences observed (p=0,935; p=0,072 and p=0,627 respectively) (**Figure 28**).



* = Extreme outlier

Figure 28. Normalized relative expression (-ΔCt) of E6, E7 and E5 in female and male anal samples.

Histologically, the only lesions observed in the anal samples of both genders were classified as LSIL with very similar incidences (90% female and 100% male LSIL anal lesions). The penetrance of HPV-induced lesions in the K14-HPV16 base of the tongue male and female lesions is described in **Table 9.**

Table 9. Spectrum of HPV-induced lesions in the K14-HPV16 anus male and female.

	Age (Weeks)	Organs	Normal	LSIL	HSIL	Invasive SCC
Female	30	Anus	10% 1/10	90% 9/10	----	----
Male	30	Anus	----	100% 10/10	----	----

4.7. Aim V

4.7.1. Correlation between lesion progression with E6, E7 and E5 mRNA expression

Our next step was to observe if there was any correlation between the grade of the lesions (LSIL and HSIL/SCC) and the expression of the E6, E7 and E5 mRNAs. This analysis was only performed for the base of the tongue samples, in the K14-HPV16 female mice, once in this tissue, all the cascade carcinogenic lesions were present. Additionally, the HSIL and SCC lesions were inserted in the same group due to the fact that HSIL is the immediate lesion right before the development of SCC. The mRNA E6, E7 and E5 expression was similar between the LSIL and the HSIL/SCC ($p=0,831$; $p=0,670$ and $p=0,522$ respectively). The results showed that the severity of lesions does not necessarily correlate with a different expression of the E6, E7 and E5 mRNAs (**Figure 29**).

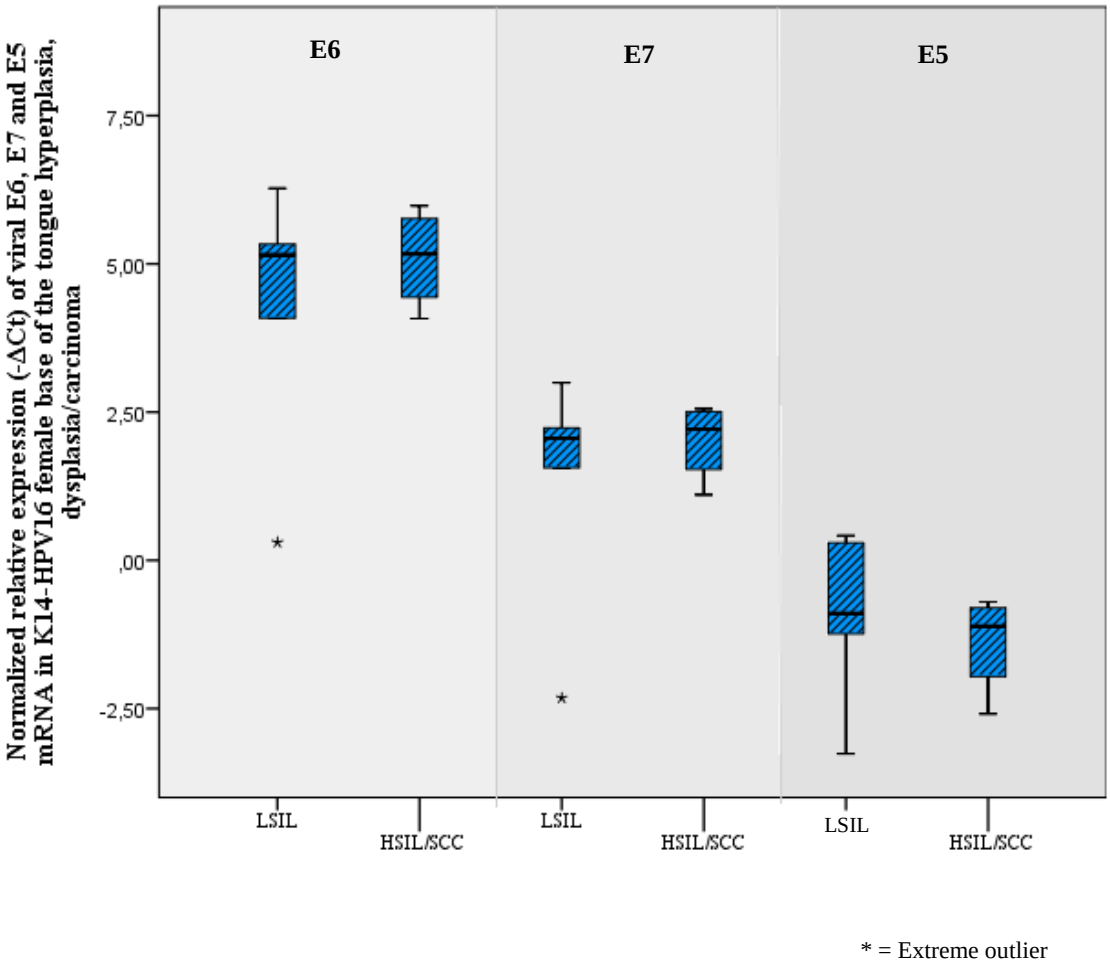


Figure 29. Normalized relative expression (-ΔCt) of E6, E7 and E5 in female base of the tongue LSIL and HSIL/SCC.

4.8. Western blot analysis

The western blot results were not available in time for this dissertation. The secondary antibody bound unspecifically to all samples at a molecular weight of approximately 25 kDa, which masked the protein signal of the E6 and E7 oncoprotein which was 23 kDa and 20 kDa, respectively. We then have tried the Protein G PLUS-Agarose Reagent (Santa Cruz Biotecnhology sc-2002) in order to clear the lysates and reduce the unspecific signal, however with no success. We have also tried several different washing steps, secondary incubation time, substrate incubation time, but none with success. The next step will be to use a conjugated primary antibody, which if specific enough will allow the visualisation of E6 and E7 oncoproteins.

Discussion

V. Discussion

5.1. *E6, E7 and E5* mRNA expression and histopathological features

Infection by HPV is considered a huge public health burden afflicting millions of people and being one of the main etiological factors for the development of cancer (1). It is responsible for the development of lesions that can progress to cancer in the reproductive, anogenital and oral anatomic sites (2). Even though the carcinogenic cascade of the cervical cancer is widely studied and understood, in the other anatomic locations infected by HPV, it is still limited. Differences in the natural history of HPV as well diverse epithelia backgrounds and tissue stromal microenvironments may play a key role in the behaviour of the HPV oncoproteins, which can potentiate different temporal, physiological and molecular actions in the different anatomic sites of infection. Understanding these differences can be crucial not only for a better knowledge of the HPV-related carcinogenic steps but also for the development of novel and more precise therapies. Nevertheless, there are main gasps of knowledge when it comes to the specific tissue's stromal microenvironment and its influence on tumour development, especially in the HPV induced-OPSCC where there is a lack of precancerous lesions characterization allied to the difficulty of having samples.

This study allows a first insight of the likely different behaviour of the HPV oncoproteins in the different anatomic sites of infection resulting in the development of a different grade of lesions in the different anatomic sites of infection. Alongside, this is the first study of the expression profile of the malignant oncoproteins E6, E7 and E5 in the several anatomic sites of HPV infection in the K14-HPV16 mouse model, that mimics the carcinogenic cascade of HPV-related cancers in humans.

Even though the expression of E6, E7 and E5 were similar in the base of the tongue and anus in both genders, the base of the tongue tissues developed more advanced lesions, namely HSIL and SCC, compared with the anus, where LSIL were mainly observed. Alongside, in the penile tissue samples, HSIL lesions were also observed, even though the transgene HPV viral mRNA expression was similar to the remaining male tissue samples. Expectably, no HPV-related lesions were observed in the uterine cervix and in the bladder samples. Overall, we verify a huge difference in the oncoproteins regulation in the different tissues. Therefore, other factors apart from the HPV mRNA transgene expression must be present in the different tissues, mediating the development of the different lesions observed. Some hypothesis apart from the pro-tumoral chronic inflammation seemed also to be important namely a differential expression of microRNA´s and hormonal receptors.

5.1.1. Implications of the anatomic site microenvironment

5.1.1.1. Base of the tongue samples

The oropharyngeal region, which comprises the base of the tongue, the soft palate, the side and back walls of the throat and the tonsils is characterized by a vast lymphoepithelial microenvironment, where a continuous spreading lymphocyte infiltrate containing immunomodulating agents is continuously produced (147,173). Even though the lymphoepithelial tissue is characterized by a fast counteraction of the immune system, functioning as a protective barrier against infection, when viral load is intense and continuous allied with the capability of the impairment of the HPV oncoproteins E6, E7 and E5 onto the immune system regulatory mechanisms, as it happens in the K14-HPV16 mouse model, it will lead to an unresolved inflammation as well as pro-tumoral phenotype transformation process which will eventually promote the a decay and decrease function of the immune system and a consequent progression of the HPV-related lesions into malignant tumours (160,174). Consequently, macrophages, mast cells, neutrophils and other innate immune cells may be chronically recruited in the already near lymphoepithelial tissues, supplying cytokines, chemokines, reactive oxygen species (ROS), growth factors and other biomolecules where together with the viral oncoproteins, can modulate a faster malignant progression by promoting a pro-tumoral phenotype of the immune cell components and increasing the occurrence of genetic and epigenetic events that are key factors for malignant progression (175–178). Additionally, studies have shown that E6 and E7 oncoproteins from HPV16 increase the transcription activity of MMP and consequently the malignancy of the HPV-related lesions, once these molecules are associated with an increase angiogenesis mechanisms and consequently lesion progression (179). Additionally, the results showed that the severity of the lesions were not influenced by a higher or lower oncogenic mRNA expression (**Figure 29**), reinforcing the existence of other internal co-factors for the development of lesions. In HPV-positive oropharyngeal cancers in humans, there are still no established co-factors identified since these types of cancers tend to appear in younger people that do not smoke or drink (180). However, they may be enhanced by some external factors such as food components (Rui M. Gil da Costa, Tiago Neto, Diogo Estêvão et al. Ptaquiloside from bracken (Pteridium spp.) promotes oral carcinogenesis initiated by HPV16 in transgenic mice. Life Sciences (submitted).

Our results suggest that in the base of the tongue, HPV oncoproteins, together with the oropharyngeal specific microenvironment, where a chronic recruitment of inflammatory cells may happen is a sufficient cause for the development of SCC.

5.1.1.2. Anal samples

In the anal samples either in K14-HPV16 male and female, the only type of lesions observed was LGSL. However, even though the expression of the HPV oncoproteins was enough to induce the first stage in the carcinogenic cascade, the time-line used in this study (30 weeks) was not sufficient to promote more advance lesions in a way of promoting additional molecular alterations for tumour progression, therefore, a longer study would be necessary to evaluate if HPV alone could promote the development of more advance lesions. Additionally, a decrease of immune pro-tumoral cells infiltration in the anal region compared with the base of the tongue could lead to a failure of lesion progression from LSIL. Furthermore, could also be interesting to study if the influence of other co-factors could reduce the time in which we would observe more advance lesions. In humans, several factors for the development of anal carcinomas have been identified. Apart from HPV, HIV infections, multiple sex partners, men who have sex with men, smoking and a consequent lower immunity seem to play important roles in the development of anal cancer (120,137).

5.1.1.3. Uterine cervix samples

In the uterine cervix, we only observed normal epithelium, with no associated lesions. The expression "per se" of the HPV oncoproteins appeared to be insufficient to induce the development of lesions in the uterine cervix. However, it is known that female K14-HPV16 mice, when exposed chronically to oestrogen, namely 17β-oestradiol, start to develop benign proliferation, ending in uterine cervix SCC in 100% of the treated mice (164). Another study led by Auborn and colleagues showed that the administration of an anti-oestrogen compound (indole-3-carbinol) could hamper uterine carcinogenesis (181) . Furthermore, this compound also seemed to reduce other highly advanced lesions commonly found in the chest skin and ear of the K14-HPV16 mice. It is, therefore, well established that HPV persistent infection is a necessary but not sufficient cause for the development of cervical cancer. The powerful role of hormones as potent and necessary co-factors that act in synergy with the HPV oncoproteins promote the malignancy development by altering the stromal tissue microenvironment in the uterine cervix which may explain why no lesions were observed with the action of the HPV oncoproteins alone (118,182).

5.1.1.4. Penile samples

In the penile tissue, we reported the development of more advanced lesions, namely HSIL. Although HSIL is the event immediately before SCC, we have not observed this type of lesion in any of the penile samples. We speculate that more time would be necessary for the development of penile cancer in K14-HPV16 male mice. When dissecting the male samples, we could observe large and inflamed inguinal lymph nodes close to the penis (Figure not shown).

As a hypothesis, we think that the specific microenvironment found near the penis, together with the viral oncoproteins could influence the progression of more advance lesions from LSIL to HSIL, similarly but not with the same influence and clearly not at the same time (30 weeks) as it might happen in the base of the tongue (183). Alongside, HPV-induced penile carcinogenesis may be enhanced by external carcinogenic compounds that are commonly found in cigarettes and can promote a faster and a more aggressive development of carcinogenic lesions. (Rui M. Gil da Costa, Diogo Estêvão et al. HPV16 induces penile intraepithelial neoplasia and penile squamous cell carcinoma in transgenic mice: a mouse model for penile cancer (paper under preparation)).

5.1.1.5. Bladder samples

Our results showed that in K14-HPV16 mice bladder tissue, the expression of E6 and E7 mRNA was residual and not present in all the samples. Alongside, there is no expression of E5 mRNA. Furthermore, the bladder histology was normal with no apparent lesions identified. HPV etiological role in the urinary tract, particularly in the bladder, has always been controversial and inconclusive (184). Studies in which HPV was found in bladder cancer showed a wide range of prevalence, from 0-80%. Actually, some researchers speculate contamination issues when HPV is detected in bladder tissues, due to HPV almost ubiquitous presence and easy contamination, leading to false positive results (185). Epithelium constraints of the bladder that is formed by the basal, intermediate and superficial (umbrella) cells allied to the non-viremic characteristic of HPV, can influence the non-successful HPV infection in the urinary tract (12). The bladder is characterized by a transitional cell epithelium, not favourable to HPV infection that needs a complete stratified epithelium to its life cycle replication (12). However, the residual mRNA expression of E6 and E7 in the bladder samples is explained by the recently discovered existence of a very small subset of basal cells that express K14 (186). (Rui M. Gil da Costa, Tiago Neto, Diogo Estêvão et al. Ptaquiloside from bracken (Pteridium spp.) promotes oral carcinogenesis initiated by HPV16 in transgenic mice. Life Sciences (submitted).

A summary of the different temporal lesions developed in the K14-HPV16 studies tissues exposed to chronic expression of HPV oncoproteins is shown in **Figure 30**.

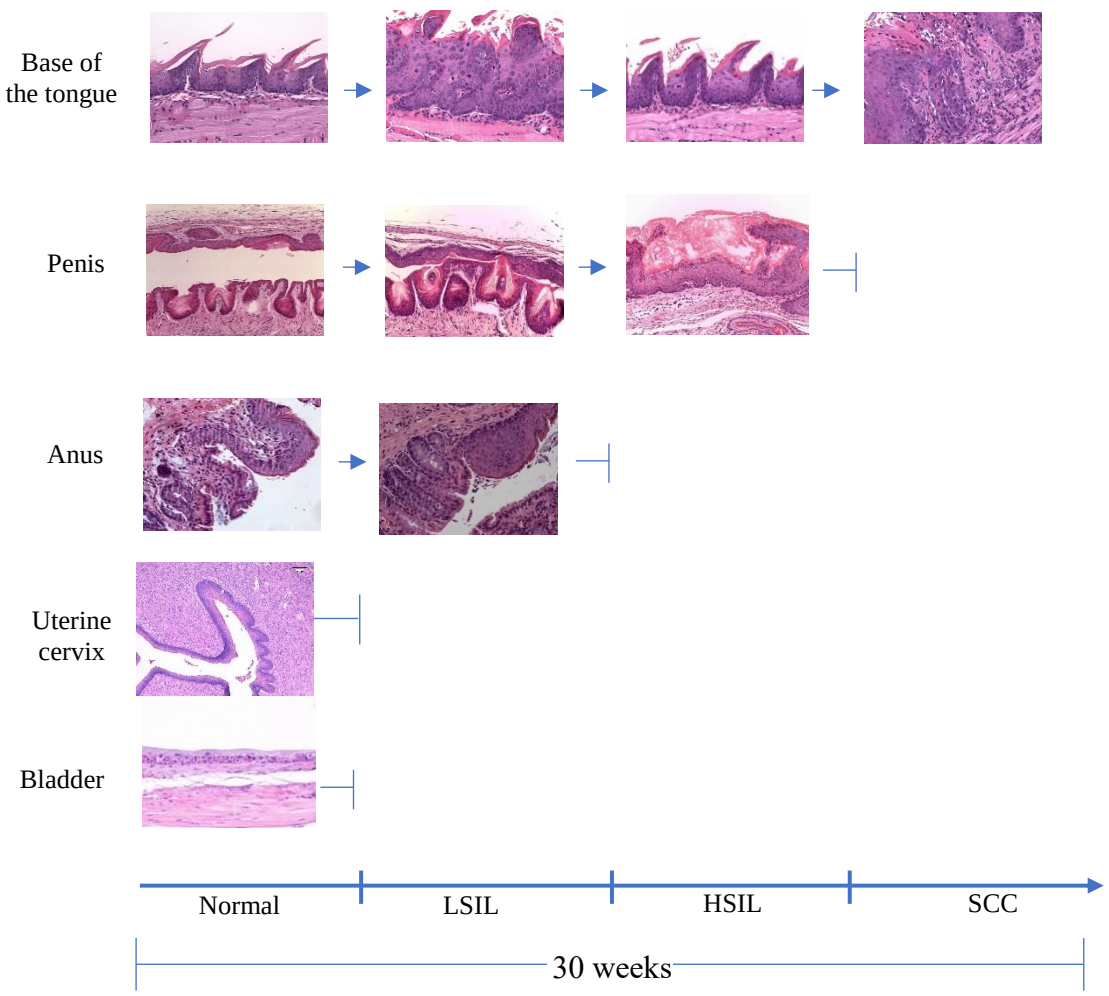


Figure 30. Carcinogenic cascade of HPV-induced lesions in the K14-HPV16 base of the tongue, penis, anus, uterine cervix and bladder.

5.1.2. Implications of the gender: Females versus males

This study revealed that in the base of the tongue there is a higher incidence of SCC in females in comparison to males (20% vs 10% respectively). Therefore, the K14-HPV16 female base of the tongue appears to be more susceptible to the progression of the malignant lesions. This susceptibility may be explained by circulating levels of steroid sex hormones, namely oestradiol and glucocorticoids, that can influence the development of such lesions in an already prone anatomic regions for the development of high advance lesions (187). The steroid sex hormones are also known to enhance the expression and behaviour of the viral oncoproteins E6, leading to a higher degradation of p53 protein and consequently inducing a more prominent carcinogenic cascade (188).

5.1.3. Expression profile of E6, E7 and E5 mRNA

Interestingly, in our study we found an almost homogenous expression profile of the E6, E7 and E5 mRNA reported in the studied tissues (E6>E7>E5).

Several mechanisms can influence the gene expression, justifying these results (189). Mechanisms of alternative splicing, polyadenylation and methylation towards the transgene cassette can interfere and change the genetic expression (189). Most mammalian mRNA undergoes alternative splicing (190). Alternative splicing is the process by which several mature mRNA sequences and protein isoforms can be achieved from the same coding gene (190). The HPV genome is polycistronic, encoding from one mature mRNA two or more proteins. Alternative splicing processes are used in order to generate different HPV mRNAs, having alternative transcription ends which can justify the cascade expression of the transgenes mRNAs (191,192). Studies have shown that there are intensive alternative splicing processes in the HPV16 genomic *E6* and *E7* regions, creating several isoforms, that have been shown to be implicated in the carcinogenesis processes of the HPV-related cancers (193,194). Alongside, E2 protein can interact with the spliceosome molecules, increasing the alternative splicing rate (195).

It is also known that in polycistronic mRNA, the first ORF, being this the case of E6 mRNA in the transgene cassette (**Figure 14**), can be translated more efficiently when intercistronic distances are short (196). Additionally, the E6 mRNA is also found to be the most abundant transcript in the carcinogenesis lesions of cervical cancer (196). This hypothesis can justify the existence of a more abundant E6 mRNA transgene transcript in comparison with the E7 and E5 transcripts (E6>E7>E5).

Other studies refer that the splicing mechanisms can be regulated by the epidermal growth factor (EGF) (196). Since E5 can promote a higher EFG activation and the K14-HPV16 is characterized by a continuous expression of this viral protein, this could lead to an increment of the alternative splicing mechanisms, leading to a higher transcription of E6 isoforms (73,197).

Chromatin conformation and RNA secondary structure also play a role in determining the efficiency of alternative splicing and which exons are spliced out or retained (190). Additionally, the promoter used in order to initiate the transcription can also influence the splicing mechanisms (198).

Concluding remarks and future perspectives

VI. Concluding remarks and future perspectives

HPV-related cancers are still a major worldwide health concern. It is, therefore, crucial to fully understand the oncogenic mechanisms involved in their development. The K14-HPV16 mouse model has an enormous potential for unravelling the HPV-related carcinogenesis steps. This knowledge may allow the development of target therapies against HPV-derived cancers, mainly anogenital and head and neck cancers that are globally increasing. This study enlightens the proof of concept of an earlier and less-external-cofactor dependent carcinogenesis induced by HPV in oropharyngeal cancers in comparison with other anatomic localizations namely the uterine cervix, the anus and even the penis, where HPV alone and taking in consideration the 30-week period of the experiment is therefore necessary but seems not to be sufficient to induce squamous cell carcinomas. This work may also recognize the stromal microenvironment especially in the oropharyngeal region as a co-factor for the development of HPV related lesions. We also conclude that due to the almost inexistent expression of E6, E7 and E5 mRNA in the bladder, allied with its transitional epithelium, it is unlikely that HPV induces bladder carcinoma. Furthermore, for the first time, the K14-HPV16 mouse model seems to be useful for the study of HPV-induced oropharyngeal cancers once it develops different grades of lesions led by the molecular action of the HPV alone.

Future studies should focus on understating the communication between the different tissue stromal microenvironments of the different HPV anatomic sites of infection and the role of the HPV oncoproteins as well as the quantification, in the different tissues of inflammatory cells that are known to be a switch from pre-cancerous lesions to malignant ones. Previous studies have shown that in the K14-HPV16 mouse model, the inflammatory cell profile was different in the epithelial skin cancer compared with the cervical cancer, once more emphasising the despair genetic, hormonal and microenvironmental regulation that happen in the different tissues even though influenced by the expression of the same oncogenes (199,200). Furthermore, it would be important to investigate the influence of external hormonal and carcinogenic compounds in the development of the lesions in the several anatomic locations of HPV carcinogenesis and its effect on the already pre-existing ones.

The oncogenic viral proteins E6, E7 and E5, being highly expressed in HPV-related lesions, are consequently one of the best targets for therapeutic applications, once they are the main drivers of high-grade lesions´ that eventually progress to cancer. While conventional therapeutic approaches such as surgery, chemotherapy and radiotherapy are the most commonly used treatment for cancer patients, tumour resistance and recurrence are frequent problems, decreasing the survival and quality of life of patients. Therefore, there is a sustained need for new and more effective cancer therapeutics.

More recently, the clustered regularly interspaced short palindromic repeats-associated protein 9, (CRISPR-Cas9), has emerged as a powerful genome-editing tool with boundless applications namely in mutation correction, inactivation of oncogenes and activation of tumour suppressor genes (201). In the future, a promising approach would be to use this technique to permanently disrupt the *E6*, *E7* and *E5* genes from the transgenic K14-HPV16 mouse genome. In another mouse model, this removal has shown to decrease p53 ubiquitination, as well as to increase cell cycle arrest, increasing the animals´ lifespan (202–204). Therefore, CRISPR-Cas9 seems to be a promising technology with therapeutic applications in HPV-related cancers (201). Estêvão D. *et al.* CRISPR-Cas9 therapies in experimental mouse models of cancer. Future Oncology. 2018 July.

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VIII. Annexes

8.1. Median, Q1 and Q3 values of E6, E7 and E5 mRNA expression

	E6_base of the tongue	E7_base of the tongue	E5_base of the tongue	E6_anus	E7_anus	E5_anus	E6_uterine cervix	E7_uterine cervix	E5_uterine cervix
Median	5,145	2,095	-0,955	4,135	2,520	-0,430	1,795	-2,440	-4,940
Q1	4,086	1,448	-1,308	3,362	1,558	-1,380	0,705	-3,998	-6,103
Q3	5,658	2,485	-0,722	5,183	3,070	0,215	5,615	1,943	-0,528

Table 10. E6, E7 and E5 values of Median, Quartile 1 (Q1) and Quartile 3 (Q3) in K14-HPV16 female mice base of tongue, anus and uterine cervix.

	E6_base of the tongue	E7_base of the tongue	E5_base of the tongue	E6_anus	E7_anus	E5_anus	E6_penis	E7_penis	E5_penis
Median	2,560	1,900	-0,720	4,200	1,550	-0,130	1,870	1,570	-1,120
Q1	1,350	0,195	-1,755	2,245	-0,865	-2,185	0,550	-0,170	-2,708
Q3	4,675	4,320	2,080	5,450	1,985	0,760	2,855	2,655	0,352

Table 11. E6, E7 and E5 values of Median, Q1 and Q3 in K14-HPV16 male mice base of tongue, anus and penis.

	E6_base of the tongue	E7_base of the tongue	E5_base of the tongue	E6_anus	E7_anus	E5_anus	E6_uterine cervix	E7_uterine cervix	E5_uterine cervix	E6_bladder	E7_bladder
Median	5,145	2,095	-0,955	4,135	2,520	-0,430	1,795	-2,440	-4,940	-6,650	-9,150
Q1	4,086	1,448	-1,308	3,362	1,558	-1,380	0,705	-3,998	-6,103	-8,120	-10,30
Q3	5,658	2,485	-0,722	5,183	3,070	0,215	5,615	1m943	0,528	-4,83	-8,30

Table 12. E6, E7 and E5 values of Median, Q1 and Q3 in K14-HPV16 female base of tongue, anus and bladder

	E6_base of the tongue female	E7_base of the tongue female	E5_base of the tongue female	E6_base of the tongue male	E7_base of the tongue male	E5_base of the tongue male
Median	5,145	2,095	-0,955	2,560	1,900	-0,720
Q1	4,086	1,448	-1,308	1,350	0,195	-1,755
Q3	5,658	2,485	-0,722	4,675	4,320	2,080

Table 13. E6, E7 and E5 values of Median, Q1 and Q3 in K14-HPV16 female and male base of the tongue.

	E6_anus female	E7_anus female	E5_anus female	E6_anus male	E7_anus male	E5_anus male
Median	4,135	2,520	-0,430	4,200	1,550	-0,130
Q1	3,362	1,558	-1,380	2,245	-0,865	-2,185
Q3	5,183	3,070	0,215	5,450	1,985	0,760

Table 14. E6, E7 and E5 values of Median, Q1 and Q3 in female and male anal samples.

	E6_LSIL	E7_LSIL	E5_LSIL	E6_HSIL/SCC	E7_HSIL_SCC	E5_HSIL_SCC
Median	5,140	2,060	-0,900	5,170	2,210	-1,115
Q1	2,245	0,590	-1,745	4,258	1,323	-2,275
Q3	5,450	2,430	0,330	5,873	2,535	0,7500

Table 15. E6, E7 and E5 Values of Median, Q1 and Q3 in female base of the tongue LSIL and HSIL/ SCC

8.2. Oral communication in the EUROGIN 2018, Lisbon, Portugal

K14-HPV16 MOUSE MODEL: A JOURNEY TOWARDS EARLY HPV-INDUCED HEAD AND NECK VS ANAL AND UTERINE CARCINOGENESIS

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Background/Objectives: Human Papillomavirus (HPV) is the most common sexual transmitted agent worldwide, being also responsible for 5 % of all human cancers. Even though cervical cancer is thought to be reducing, HPV positive anogenital and head and neck cancers are regrettably increasing. Differences in the natural history of HPV have been observed by gender and anatomic sites of infection where epithelial backgrounds and tissue microenvironment may play a crucial role. The main goal of this study was to understand if E6, E7 and E5 oncoproteins may promote distinct roles in the carcinogenesis cascade in K14-HPV16 mice model base of tongue, anus and uterine cervix characterized by different tissue microenvironments. Methods: The base of the tongue, anus and uterine cervix samples were collected from 10 female 30-week-old K14-HPV16 transgenic mice. Histopathological analysis of the tissues was performed for tissue characterization. Tissue samples were classified as normal, hyperplasic, dysplastic and carcinoma. The E6, E7 and E5 mRNA levels were quantified by real-time PCR being normalized by a combination of the best two housekeeping genes. Statistical analysis was performed using the IBM SPSS Statistics (Version 24.0). Mann-Whitney tests were used to evaluate statistical differences in normalized relative expression (–ΔCt) of the E6, E7 and E5 genes among the different tissue samples. Results: We observed a higher incidence of more advanced lesions, namely HSIL and carcinoma on the base of the tongue tissue samples in comparison with the anus where all lesions were hyperplasic. All the uterine cervix samples presented to be normal. Furthermore, the expression of the oncogenic HPV viral mRNA was

detected across tissues with no significant overexpression within the different lesions. Conclusion: This study enlightens the proof of concept of an earlier and less-cofactor dependent carcinogenesis induced by HPV in oropharyngeal cancers in comparison with other anatomic localizations. In the base of the tongue, cancer was induced within the mice 30 weeks period, in comparison with the anus and uterine cervix, where HPV itself seems not to be sufficient to promote advanced lesions even though the expression of the viral mRNA´s are detected and similar within the tissues. Future studies should focus on understanding the behaviour of the HPV oncoproteins and the related oncogenic pathways at multiple anatomic locations of infection, representing different tissue microenvironments. This might allow a better understanding of tissue-specific HPV-related carcinogenic steps and a consequent precision therapy.

8.3. Poster presentation in the Aims Meeting 2019, Lisbon, Portugal

K14-HPV16 MOUSE MODEL: A JOURNEY TOWARDS EARLY HPV-INDUCED HEAD AND NECK VS ANAl, UTERINE AND BLADDER CARCINOGENESIS

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Introduction: Differences in the natural history of HPV have been observed in the anatomic sites of infection, where epithelial backgrounds and tissue microenvironments may play a crucial role. The main goal of this study was to observe if E6, E7 and E5 HPV oncoproteins trigger distinct carcinogenic pathways in the K14-HPV16 mouse model, on the base of tongue, anus, uterine cervix, penis and bladder, which are characterized by different tissue microenvironments and epithelia. Methods: Samples from the base of the tongue, anus, uterine cervix and bladder were collected from ten K14-HPV16 female mice 30-week-old. The E6, E7 and E5 mRNA levels were quantified by RT-PCR, after normalization using the best two housekeeping genes. Histopathological analysis of the tissues was performed for tissue characterization and classified as normal, hyperplastic, dysplastic and carcinoma. Statistical analysis was performed using the IBM SPSS Statistics. Kruskal-Wallis tests were used to evaluate statistical differences in normalized relative expression of E6, E7 and E5 Results: The expression pattern of the oncogenic HPV viral mRNAs E6, E7 and E5 was similarly detected across the tissues ($p>0,05$), except in the bladder ($p< 0.01$). However, we observed a higher incidence of more advanced lesions, namely dysplasia and squamous cell carcinoma in the base of the tongue when compared with the anus, where hyperplasia was the most observed lesion. In uterine cervix and bladder samples, only normal epithelium was observed. Alongside, no significant difference of the transgene's HPV mRNAs E6, E7 and E5 expression were found within the three types of lesions that were characterized in this study ($p=0,831$, $p=0,670$ and $p=0,522$ respectively).

Conclusion: This study suggests that the carcinogenesis induced by HPV in oropharyngeal cancers is less dependent on co-factors, compared with the other anatomic locations. In the base of the tongue, cancer was induced within the mice 30 weeks period, in comparison with the other anatomic locations, where HPV is not sufficient to promote advanced lesions even though the expression of the viral mRNAs was detected similarly within the tissues. Future studies should focus on understanding the behaviour of the HPV oncoproteins at multiple anatomic locations of infection, representing different tissue microenvironments. This might allow a better understanding of tissue-specific HPV carcinogenesis and the development of precision therapies.

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